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***CO-ORDINATE REGULATION OF ANTIBIOTIC
AND PIGMENT PRODUCTION BY THE Serratia
RAP PROTEIN: EVIDENCE FOR A NOVEL FAMILY
OF REGULATORY PROTEINS***

By

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SUMMARY

The enteric bacterium *Serratia marcescens* is an opportunistic human pathogen. The strain studied here makes the red pigment prodigiosin (Pig) and the β -lactam antibiotic (5R)-carbapen-2-em-3 carboxylic acid. Mutants were isolated which were affected for pigment production. Approximately 20% of these mutants were also concomitantly deficient for the production of antibiotic. These mutants were presumed to be defective in the *rap* (regulation of antibiotic and pigment) gene. This study set out to investigate the *rap* gene which had been cloned by direct cosmid complementation of a Rap mutant from a cosmid library (pNRT300). Sequence analysis of the *rap* gene revealed a predicted product showing strong homology to SlyA, classified by Libby *et al.*, (1994) as a virulence determinant in *Salmonella*. Homologues of the *rap* gene were detected in several genera including *Salmonella*, *Yersinia*, *Enterobacter* and species of the enteric plant pathogen *Erwinia*. The *Erwinia hor_{Ec}* gene (homologue of *rap*) was cloned and encoded a product which was highly homologous to both the SlyA and Rap proteins. The gene arrangement around the *rap* locus in *Erwinia* was identical to that in *Serratia* in that *rap* and *hor_{Ec}* were both situated upstream of two genes encoding homologues of the lipoprotein Pcp and a gene encoding a protein of unknown function from *Yersinia enterocolitica*. This observation led to the search for the *Yersinia* homologue of *rap* (*hor_{Ye}*) which was subsequently cloned and sequenced. This gene too encoded a protein highly homologous to Rap and Hor_{Ec}. Data base searches revealed that these proteins shared a significant level of homology with a number of bacterial protein regulators involved in exoenzyme production, virulence in plant and human pathogens, multiple antibiotic resistance and xenobiotic catabolism. The findings of this study cast serious doubt on the conclusions of Libby *et al.*, (1994) and in a recent report which was published whilst this thesis was being compiled, Ludwig *et al.*, (1995) reclassified SlyA as a regulatory protein capable of activating cryptic haemolysin genes in *Escherichia coli*.

Marker exchange mutants (*hor_{Ec}::kan^R*) of the *Erwinia carotovora* subspecies *carotovora* were found to be affected in the production of a carbapenem antibiotic and showed decreased levels of production of multiple exoenzyme virulence factors. Transcriptional fusion data revealed that the *hor_{Ec}* mutation affected the transcription of *carA* a carbapenem biosynthetic gene. Antibiotic and exoenzymes are known to be regulated by a small molecule dependent regulatory system analogous to the *Lux* system controlling bioluminescence in *Photobacterium fischeri*. The results of regulatory studies in which autoinducer was added exogenously, or *carR* was added *in trans* imply a role for Hor_{Ec} in this pheromone-signalling system.

The functional expression of prodigiosin in a *Erwinia carotovora* subspecies *carotovora* was found to be dependent on autoinducer and the gene product of *hor_{Ec}*. Some interesting observations were also made regarding differential patterns of prodigiosin gene expression within bacterial colonies. These patterning effects were strikingly strain-specific.

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My last words are reserved for Angie and my family (past and present). Knowing that I owe much of this to them I would like to dedicate this thesis:-

To Angie and My family

Thanks!

Let justice be done though the heavens may fall!

(Author unknown)

DECLARATION

This thesis has been compiled by myself and has not been used in any previous application for a degree. The results were obtained by myself, except where the contributions of others have been acknowledged. All sources of information have been specifically acknowledged by means of references.

ABBREVIATIONS

A	adenosine	IPTG	isopropyl β -D-thiogalactosidase
ATP	adenine tri-phosphate	Kb	kilo base pair
bp	base pair	KDa	kilo Dalton
C	cytosine	l	litre
Cel	cellulase	M	molar
CIP	calf intestinal phosphatase	mg	milligramme(s)
cm	centimetre	min	minutes
C-terminal	carboxy terminal	ml	millilitre
Da	Daltons	mM	millimolar
dATP	2'-deoxyadenosine 5'-triphosphate	MW	molecular weight
dCTP	2'deoxycytidine 5'-triphosphate	NB	nutrient broth
dGTP	2' deoxyguanosine 5'-triphosphate	NBA	nutrient broth agar
HSL	<i>N</i> -(3-oxohexanoyl)-L-homoserine lactone	nm	nanometres
dITP	2'deoxyinosine 5'-triphosphate	N-terminal	amino-terminal
DIG	digoxigenin	PEG	polyethylene glycol
DMF	dimethyl formamide	Pel	pectate lyase
DNA	deoxyribonucleic acid	PGA	polygalacturonate
dNTP	2' deoxynucleotide 5'-triphosphate	Prt _R	protease Resistant
EDTA	diaminoethanetetra-acetic acid	rpm	revolutions per minute
EMS	ethyl methane sulphonate	SD	ribosome binding site (Shine and Dalgarno)
g	gramme(s)	SDS	sodium dodecyl sulphate
G	guanine	T	thymine
hrs	hours	OD	optical density
		OHHL	<i>N</i> -(3-oxohexanoyl)-L-homoserine lactone
		Orf	open reading frame

Orf	open reading frame
PCR	polymerase chain reaction
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA
TEMED	N,N,N',N-tetramethylethylenediamine
Tris	2-amino-2(hydroxymethyl)-1-3- propane diol
U	uracil
µg	microgrammes
µl	microlitres
v	volt
w/v	weight/volume
X-Gal	5-bromo-4-chloro-4-indolyl β-D-galacto pyranoside

AMINO ACID ABBREVIATIONS

A	alanine	M	methionine
C	cysteine	N	asparagine
D	aspartic acid	P	proline
E	glutamic acid	Q	glutamine
F	phenylalanine	R	arginine
G	glycine	S	serine
H	histidine	T	threonine
I	isoleucine	V	valine
K	lysine	W	tryptophan
L	leucine	Y	tyrosine

INTRODUCTION

1.0 INTRODUCTION

1.1 THE HISTORY OF A GREAT BACTERIUM

The history of the genus *Serratia* has been traced back to antiquity (reviewed by Gaughran, 1969; Cullen, 1994). It is thought that the growth of *Serratia* on starchy foods such as polenta and sacramental bread (Host) has for countless years been mistaken for blood. Perhaps the earliest report of this kind was given by Quitus Curtius Rufus, a Roman Historian writing in the 1st century AD (cited in Gaughran, 1969). He wrote about Alexander the Great who in 332 BC lay siege to the Lebanese city of Tyre (a centre of commerce and an important naval base) for seven months under a constant barrage of arrows and rocks. The Macedonian soldiers were becoming disillusioned with their mission. When they broke open their bread ration they found blood inside!. At first this was taken to be an onerous portent. However a skilled Seer named Aristander is said to have declared that, because the blood came from within, it foretold of impending doom for those within the fortress and victory for the Macedonians. This gave encouragement to the weary troops and when the city did eventually fall 8,000 Tyrians were killed and 30,000 were sold into slavery.

Another historically important event that could be attributed to *Serratia* took place in Italy in 1263 AD in a small village called Bolsena (Ehrenberg, 1848; cited in Cullen, 1994). Whilst blessing the elements of Holy communion and at the same time doubting whether the Host truly became the body of Christ, a priest saw drops of blood run from the Holy bread and run onto his robe. He attempted to clean his hands and robe but only succeed in staining the alter and its cloth. He was so taken by this event that he immediately sought absolution for his doubts from Pope Urban IV, who was resting in a nearby village. Following this event, in 1264 AD, Pope Urban IV is said to have issued a bull instituting the feast of Corpus Christi in the memory of this miracle at the mass of Bolsena. The event was further immortalised by Raphael who painted a fresco in the Vatican, inspired by this Miracle, called The Mass of Bolsena (figure 1.1).

FIGURE 1.1



Figure 1.1 The Mass of Bolsena. A fresco in the *Stanza d'Elodoro*, The Vatican, Rome. Painted by Raphael

There are other documented instances where the appearance of droplets of blood on the religious Host have been presumed to be because of the desecration of the consecrated wafers by heretics and non-believers. One such example took place in Nuremberg in 1298 AD, where hundreds of Jews were slaughtered because a stolen Host, which had been ground up in a mortar and pestle, was seen to spill blood (Batman, 1581; cited in Gaughran, 1969). The unexplained appearance of blood is not restricted to antiquity. In 1958 a child born in a hospital in Wisconsin was reportedly suffering from 'Red Diaper Syndrome' (Waisman, 1958; cited in Gaughran, 1969). The child's stools, urine and newly soiled nappies appeared normal. However nappies stored before washing developed a red blood like coloration. This totally baffled the Hospital staff until the child's stools were eventually cultured and a chronic asymptomatic *Serratia marcescens* infection was identified.

One of the first scientists to analyse the blood like substance found growing on starchy food was Bartolomeo Bizio. Between 1819 and 1824 he published reports on the nature of this phenomenon. After microscopic analysis he concluded that the red slime was a living organism described as small stemless fungi clustered in a semispherical capsule containing first pink then red liquid (Cullen, 1994). Bizio named the genus *Serratia* in honour of the Italian physicist Serafino Serrati who designed the first steam boat, and according to Bizio had been much neglected. The species name *marcescens* in Latin roughly translates as - to wither or decay. The genus name is one of the oldest in scientific nomenclature (Gaughran, 1969). It is important to note, as Gaughran concedes, there are many other explanations for the appearance of "blood" on the Host in classical and medieval times. However, if the appearance of blood was the result of bacterial growth, then although this bacterium is an opportunistic human pathogen, because of its pigment, it has probably caused the deaths of more people than many pathogenic bacteria!.

1.2 THE GENUS *Serratia*

The number of species assigned to the genus *Serratia* has been rather confused (Grimont *et al.*, 1977; Grimont and Grimont, 1978). At the beginning of this century there were 76 species included in this genus, primarily because of the ability to produce a red or pink pigment. More recently, using more complex analytical techniques this has been reduced to ten (excluding subspecies): *S.marcescens*, *S.liquifaciens*, *S.proteomaculans*, *S.plymuthica*, *S.rubidaea*, *S.odorifera*, *S.grimesii*, *S.ficaria*, *S.fonticola* and *S.entomophila* (Grimont and Grimont, 1984).

The genus *Serratia* are Gram-negative members of the Enterobacteriaceae. They are characterised by short non-spore forming rods which are motile, with the aid of peritrichous flagella. Members of the genus are facultatively anaerobic and will grow avidly between 20-37°C (Grimont and Grimont, 1984). *Serratia* species are ubiquitous in the environment: air, soil, fresh water and marine environments. They are also important pathogens of insects and fish, and are opportunistic pathogens of animals and humans. The distribution and ability to cause disease varies wildly between species and even biotypes (Grimont and Grimont, 1978). The habitats that *Serratia* species have been isolated from include coconuts, grass, mushrooms, commercial vegetables and salad crops (Grimont and Grimont, 1978).

There has been extensive literature on the isolation of *Serratia* species from insects. However they are not considered important pathogens of insects in the environment, but *Serratia liquifaciens* is known to be virulent pathogen of reared insects and can cause lethal septicaemia after penetration into the haemocoel. Poultry have also been found to harbour *Serratia* in their digestive tract, which can lead to infection of eggs, death of the embryos, and contamination of carcasses (Grimont and Grimont, 1978).

Until the latter part of this century the isolation of *Serratia* species from humans was rare. Indeed, at this time, the genus was considered harmless and, because of its

characteristic red pigment, *S.marcescens* was used as a trace organism in various clinical experiments (Simberkoff, 1980). Early workers used *S.marcescens* to investigate bacteremias after tooth extraction. The teeth and gums were smeared with a gauze soaked in bacteria. Following the removal of the tooth, blood samples were taken and cultured for *Serratia*. Surprisingly, when recent evidence is considered (discussed below) no subjects were said to have suffered any direct ill effects from this type of experiment. One possible explanation for this is derived from the observation that most clinically isolated *Serratia* species are non-pigmented and in general where pigmented *Serratia* species have been isolated from patients, the infection was asymptomatic (Williams and Quadri, 1980). The significance of this observation has not been established.

More recently *Serratia* species have emerged as significant causative agents of nosocomial infections, most notably in the urinary and respiratory tracts and infections of the blood and wounds. The category of patients most at risk are infants, the elderly and the immunocompromised (Daschner, 1980; Von Graevenitz, 1980). The majority of human infections are caused by *S.marcescens* and *S.liquifaciens* (Daschner, 1980), although there have been several reports of *S.rubidaea* and *S.odorifera* as invasive pathogens and *S.plymuthica*, *S.proteomaculans* and *S.fonticola* causing catheter infections, fatal pneumonia and leg abscesses respectively (Ursua *et al.*, 1996; Chmel, 1988; Horowitz *et al.*, 1987; Bollet *et al.*, 1993; Bollet *et al.*, 1991). These species are however seldom isolated from clinical specimens and their role as causative agents of human disease is equivocal.

In keeping with the number of environments *Serratia* species inhabit, they have been found to produce an array of extracellular proteases (serine protease and metalloprotease), bacteriocins, nucleases, lipases and chitinases (Yanagida, 1986; Braun and Schmitz, 1980; Clegg and Allen, 1985; Hines, 1988). Motility in its various forms has also been shown to be an important factor in virulence and colonisation of catheters and prosthetic devices inserted into the human body (Daschner, 1980).

Serratia can spread through or on media by a number of processes. So-called “Swimmer” cells are characterised by short rods (0.5-2µm long) with one or two polar flagella. Swarming motility predominates if there is an increase in microviscosity. Under these circumstances cells undergo morphogenesis by elongating (5-30µm) and becoming hyperflagellated (Alberti, 1990). Several *Serratia* species have also been found to make a ‘wetting agent’ which facilitates the third form of motility; spreading motility. This form of translocation unlike swimming and swarming is thought to be a passive process (Matsuyama, 1986) involving the production of a surfactant called Serrawettin (cyclo-[D-3-hydroxydecanoyl-L-seryl]₂; Matsuyama *et al.*, 1995).

Serratia is perhaps most well known for the production of the red pigmented, secondary metabolite, prodigiosin (discussed in section 1.6; Williams and Quadri, 1980). The *Serratia marcescens* strain used in this study (ATCC39006; referred to as *S.marcescens* from this point onwards) also produces another secondary metabolite, the β-lactam antibiotic, carbapenem. The following sections will look at regulatory mechanisms controlling the production of a small number of well characterised secondary metabolites, in an attempt to illustrate several themes in their regulation, before finally focusing on the antibiotic and pigment production by *S. marcescens*.

1.3 SECONDARY METABOLITES

There has been and still is a great deal of debate surrounding the true nature of secondary metabolites. The first person to define secondary metabolites was Bu’Lock, (1961). Bu’Lock considered secondary metabolites to be distinguished from ‘general’ metabolism (primary metabolism) by having a restricted distribution and no obvious function in general metabolism. The biosynthesis of these products was considered simply to act as an overflow to reduce high concentrations of normal cellular constituents which accumulate at a time in the growth cycle when primary metabolic activity is low, i.e. idiophase. There is supporting evidence for this: undecylprodigiosin produced by *Streptomyces coelicolor* A3(2) has been reported to act as a sink for

proline. Wasserman *et al.*, (1974) showed that proline was a precursor of undecylprodigiosin and Hood *et al.*, (1992) reported that mutants unable to catabolise proline, overproduced undecylprodigiosin. Analysis of the mutants revealed that although degradation of proline was defunct, biosynthesis remained unaffected, with the excess proline being shunted into the production of pigment. This observation is thought to reflect the natural function of proline as an osmoprotectant and undecylprodigiosin as the sink removing excess proline from the system (reviewed by Hopwood *et al.*, 1995).

Williams *et al.*, (1989b) do not share the view that secondary metabolites have been maintained as a shunt, processing the by-products of primary metabolism. They propose that the only definition that adequately explains the current knowledge of secondary metabolites is one which encompasses the notion that these natural products confer a selective advantage on the producing organism. They propose that secondary metabolites are '*natural substances which do not play an explicit role in the internal economy of the organism that produces it*' and that they have evolved '*because they confer certain selective advantages by interacting with specific receptors in other organisms, which may result in death or disablement of the target organism*'. Evidence cited to backup their theory includes: the majority of organisms that produce secondary metabolites lack an immune system and so secondary metabolites act as an alternative defence mechanism. In addition, it has been observed that the genes involved in antibiotic synthesis are usually very complex and almost always clustered. If there were no selective advantage for these natural products it is difficult to see why, if they have arisen through spontaneous mutation events and gene duplications, there is very little 'junk' and unrelated DNA within the cluster (Stone and Williams, 1992; Maplestone *et al.*, 1992). The idea that secondary metabolites confer a selective advantage to the producer is also shared by Kell *et al.*, (1995) who affirms that the fact that no function has been assigned to some natural products is because of a lack of suitable assay procedures to detect their activity.

Davies (1990) addresses the question of the physiological or ecological role of secondary metabolites from a very different viewpoint, arguing that the generally accepted view that antibiotics are defense mechanisms, is an anthropomorphism. The essence of his theory is that secondary metabolites are chemical fossils, structures originated in a pre-protein era formed by spontaneous condensation reactions. These molecules are said to have played an important part in the development of early life forms as primitive effectors or co-factors. As biochemical evolution progressed these molecules were replaced by the much more efficient polypeptides, however, their continued activity results from the persistence of ancient binding sites. This is in contrast to the theory of Williams *et al.*, (1989) in which secondary metabolites have evolved over history to interact with existing receptors involved in a diverse set of physiological processes.

It appears that secondary metabolites, although easily identified, are difficult to define. The only common feature identifying secondary metabolites appears to be that they are superfluous to the primary metabolic activities that allow the organism to grow and multiply. As to their function, the dearth of theories and evidence relating to the function of secondary metabolites prompted Demain, (1974) to write '*analysis of the problem of function would probably proceed faster if we stopped attempting to formulate a grand and glorious unified proposal to explain the function of all secondary metabolites*'.

The focus of this study centres on the regulation of the secondary metabolites prodigiosin and carbapenem produced by *Serratia marcescens*. Before talking more specifically about their production it may be beneficial to briefly consider the breadth of those natural substances deemed to be secondary metabolites and, with specific examples, to demonstrate general themes in their regulation.

1.4 THE FUNCTION OF SECONDARY METABOLITES

Whether one considers secondary metabolites as shunts from primary metabolism, or as natural products conferring a selective advantage on the producer organism, many of these natural products have fascinating biological properties (for a review see Vining, 1990).

Perhaps the most intensively studied secondary metabolites are those which act as antagonists against other organisms, such as penicillin or phaseolotoxin, an antimetabolite produced by *Pseudomonas syringae*. Although the majority of research has focused on this class of secondary metabolite, there is an extensive array of others. Metal transport agents called siderophores are also considered secondary metabolites (Demain, 1995) and are usually essential for virulence of invasive pathogens in the iron deplete conditions of the host. Siderophores can also be of benefit to man. The pseudobactin producing *Pseudomonas fluorescens*, is used as a seed inoculant promoting plant growth by suppressing the growth of other, often phytopathogenic, bacteria and fungi (Demain, 1995).

Various stages of sporulation have also been found to involve the production of secondary metabolites. Tyrocidine, an antibiotic produced by *Bacillus brevis* (see section 1.5.1), can actually induce sporulation under certain conditions. Secondary metabolites are also important in maintaining dormancy of the spores. Gramicidin S, produced by *Bacillus brevis*, is thought to perform a multitude of functions. It is an inhibitor of the latter stages of germination, it is hydrophobic, a property which may aid in the dispersal of the spores and it has antimicrobial properties which are proposed to protect the spores (Demain, 1995).

Knowledge of the regulatory mechanisms that are involved in the production of some secondary metabolites is quite well developed and will be discussed in the following section.

1.5 REGULATORY SYSTEMS FOR THE INDUCTION OF SECONDARY METABOLISM

Broadly speaking, when an organism is growing rapidly under optimal conditions secondary metabolism is suppressed. Only when growth rate declines as nutrients are depleted does secondary metabolism become active. In culture, this will produce the classic biphasic growth essentially partitioning primary and secondary metabolism. Doull and Vining, (1995) argue that the separation of primary from secondary metabolism should not be used as a defining characteristic, because the onset of secondary metabolism is a function of growth rate. This is illustrated by cells which are grown from inoculation in nutrient deplete media. Under these circumstances cells never enter a period of rapid growth and the formation of products like antibiotics parallels growth.

Many of the early studies on the regulation of secondary metabolism centred on the effect of environmental factors such as temperature and pH. In the main, the direct regulatory mechanisms that mediate the effects of these external factors are unknown. Prodigiosin, produced by *S.marcescens*, was one of the earliest model systems for studying the production of secondary metabolites and so these environmental factors will be discussed with respect to prodigiosin in section 1.6.4. Only recently has our attention focused directly on the genetic and biochemical systems for the regulation of secondary metabolism (for a review see Doull and Vining, 1995). The remaining part of this section will focus on genetic and biochemical systems which have been shown to regulate secondary metabolism. Several well characterised systems from a number of bacteria will be used in an attempt to illustrate the diversity of the regulatory mechanisms that govern their production. These will include regulation of microcins, the production of the tyrocidine antibiotic by *Bacillus* species, pathway and global regulators found to activate the production of undecylprodigiosin in *Streptomyces*, and finally the rapidly expanding area of bacterial pheromones illustrated by the *Photobacterium fischeri* paradigm.

1.5.1 TYROCIDINE PRODUCTION BY *Bacillus*: COMMON REGULATORY ELEMENTS BETWEEN ANTIBIOTIC PRODUCTION AND MORPHOLOGICAL DIFFERENTIATION

An excellent example of the complex interplay between proteins which regulate secondary metabolism and sporulation has been unravelled in the genus *Bacillus* (reviewed by Hoch, 1993). *Bacillus brevis* produces a variety of antibiotics and enzymes in the transition period between vegetative growth and sporulation. The regulatory hierarchy for their production centres on the gene product of *abrB*. AbrB is a transition phase regulatory protein which is produced during vegetative growth and represses the activity of a series of genes that are associated with stationary phase or are not required during favourable growth conditions (Strauch *et al.*, 1989; Strauch and Hoch, 1993; summarised in figure 1.2a). One of these genes is *tycA* which encodes the enzyme tyrocidine synthetase I, responsible for the first stage in the production of the decapeptide antibiotic tyrocidine (Marahiel *et al.*, 1987), which is thought to protect the sporulating cell. AbrB represses the activity of various other genes including sporulation genes such as *spo0H*, *spoVG*, *spoOE* and *aprE* (encoding alkaline protease; see Furbass, 1991). In addition AbrB also activates the transcription of *hpr* (Perego and Hoch, 1988), the product of which is itself a repressor of alkaline protease (encoded by *aprE*; Ferrari *et al.*, 1988) and neutral protease production (encoded by *nprE*; as shown in figure 1.2a; Strauch *et al.*, 1989; Kallio, 1991).

AbrB binds to specific sites within the promoter region of genes in the AbrB regulon, thereby preventing transcription (Robertson *et al.*, 1989; Furbass *et al.*, 1991; reviewed by Strauch and Hoch, 1993). Binding is co-operative with AbrB forming multimers, the strength of binding increasing with increased cellular AbrB concentration. Expression of the *abrB* gene is regulated by proteins involved in sporulation. Sporulation is initiated by an unknown signal, associated with the depletion of nutrients. This unknown signal induces the autophosphorylation of KinA, the first protein in a phosphorelay cascade ending with the phosphorylation of Spo0A to give

FIGURE 1.2

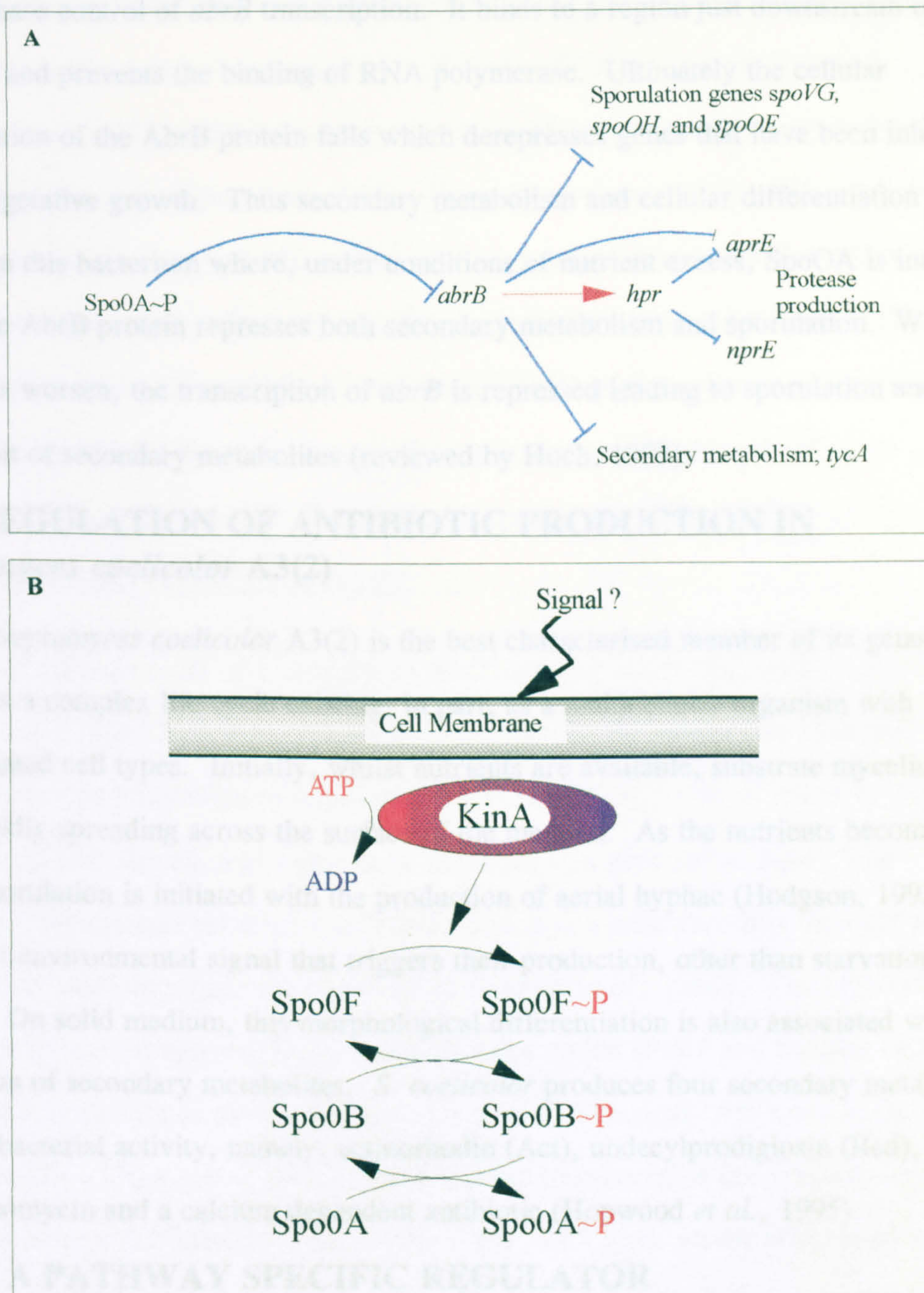


Figure 1.2

(A) A model summarising the regulatory network that surrounds the *abrB* gene. The gene product of *abrB* is a transition state regulator which during vegetative growth represses or activates the transcription of various genes (see text for details). The red line represents activation, the blue lines represent repression. The activated *Spo0A ~ P* protein (see **B**) represses the transcription of *abrB* thereby derepressing genes in the *AbrB* regulon.

(B) The sporulation phosphorelay system from *Bacillus subtilis*. An unknown signal associated with nutrient depletion activates the kinase *KinA*. *KinA* provides the major phosphate input to *Spo0F* which in turn is substrate for *Spo0B*. *Spo0B ~ P* then provides the phosphate for *Spo0A*. *Spo0A ~ P* is a transcriptional activator responsible for the regulation of multiple genes, including *abrB*.

SpoOA ~ P (as summarised in figure 1.2B). Activated SpoOA ~ P is responsible for the growth phase control of *abrB* transcription. It binds to a region just downstream of its promoter and prevents the binding of RNA polymerase. Ultimately the cellular concentration of the AbrB protein falls which derepresses genes that have been inhibited during vegetative growth. Thus secondary metabolism and cellular differentiation are coupled in this bacterium where, under conditions of nutrient excess, SpoOA is inactive and so the AbrB protein represses both secondary metabolism and sporulation. When conditions worsen, the transcription of *abrB* is repressed leading to sporulation and the production of secondary metabolites (reviewed by Hoch, 1993).

1.5.2 REGULATION OF ANTIBIOTIC PRODUCTION IN *Streptomyces coelicolor* A3(2)

Streptomyces coelicolor A3(2) is the best characterised member of its genus. It undergoes a complex life cycle existing, in part, as a multicellular organism with differentiated cell types. Initially, whilst nutrients are available, substrate mycelium grow rapidly spreading across the surface of the medium. As the nutrients become scarce sporulation is initiated with the production of aerial hyphae (Hodgson, 1992). The exact environmental signal that triggers their production, other than starvation, is unclear. On solid medium, this morphological differentiation is also associated with the production of secondary metabolites. *S. coelicolor* produces four secondary metabolites with antibacterial activity, namely; actinorhodin (Act), undecylprodigiosin (Red), methylenomycin and a calcium dependent antibiotic (Hopwood *et al.*, 1995).

1.5.2.1 A PATHWAY SPECIFIC REGULATOR

The regulatory systems for the production of Red are quite well understood (see Hopwood *et al.*, 1995). The Red cluster has been cloned and partially sequenced (Malpartida *et al.*, 1990) and a pathway specific regulatory gene has been identified; *redD* (Narva and Feitelson, 1990). RedD was subsequently defined as a positive acting

regulatory protein by:- i) the inability of *redD* mutants to co-synthesise undecylprodigiosin with other *red* mutant classes (Rudd and Hopwood, 1980; Feitelson *et al.*, 1985) ii) the lack of expression of *redE* and *redF* genes in a *redD* mutant (Feitelson *et al.*, 1985) and iii) the over expression of undecylprodigiosin when *redD* is in multicopy (Narva and Feitelson, 1990). From the sequence of the *redD* gene, the predicted protein product was 350 amino acids long, containing no DNA binding motifs. Database searches failed to find any significant similarities from other bacteria (Narva and Feitelson, 1990), although the N-terminus of RedD shows a degree of similarity with that of ActII-Orf4 the pathway specific regulatory protein for actinorhodin production (Takano *et al.*, 1992; Fernandez-Moreno *et al.*, 1991).

1.5.2.2 GLOBAL REGULATORS

Bald mutants (Bld) of *S. coelicolor* A3(2) are also interesting with respect to the regulation of Act and Red. Bld mutants are defective for the production of aerial mycelium and the production of antibiotics (Merrick, 1976). One of the best characterised Bld mutants was found to be defective in a gene called *bldA*. The sequence of the mutants revealed that it had a lesion in a gene encoding the only leucine tRNA which could recognise the codon UUA (Lawlor *et al.*, 1990). The high G+C DNA content of *Streptomyces* DNA means that this codon is rare and because of this the UUA codon is thought to have a regulatory function. The *actII*-Orf4 open reading frame contains a UUA codon which means that the translation of this transcript is *bldA* dependent (Fernandez-Moreno *et al.*, 1991). The *redD* gene, on the other hand has no UUA codons. However, the transcription of this gene was also found to be *bldA* dependent. The reason for this was thought to be that a regulatory factor which activates *redD*, higher in the Red regulatory cascade, must be *bldA* dependent. Such a factor may have been identified, encoded by the *redZ* gene (C. Flaxman, pers comm). Whilst Act and RedD production is affected by the *bldA* gene product, there have been conflicting reports concerning the temporal expression of *bldA*. Gramajo *et al.*, (1993)

found that *bldA* transcripts were present throughout growth, yet other researchers have reported that they increase during growth (Leskiw *et al.*, 1993) and so the exact role *bldA* fulfils is unclear.

Other global intracellular signals have been implicated in the induction of antibiotic production. One of these global triggers is the highly phosphorylated guanine residue, ppGpp (Ochi, 1990). In *E.coli* this molecule is part of a complex series of regulatory events known as the stringent response (reviewed by Cashell and Rudd, 1987). The signal initiating the stringent response is the presence of uncharged tRNA molecules, brought about by declining concentrations of amino acids, usually in the latter stages of the growth cycle. The net result of this response is a general reduction in the synthesis of stable RNA (rRNA and tRNA), a reduced growth rate and the induction of stationary phase gene expression through stringent promoters. In *Streptomyces*, like *E.coli*, the production of ppGpp is also coincident with a reduction in the growth rate. There have been several conflicting reports relating to the significance of ppGpp in the induction of antibiotic production. Takano *et al.*, (1992) report that the production of Red is more closely correlated with a reduction in growth rate than the occurrence of ppGpp. This view is also shared by Chakraborty *et al.*, (1996). Their results have shown that ppGpp is not essential for the production of Act but under certain conditions can influence the onset of its production. Other global regulators known to affect antibiotic production in *Streptomyces* are the γ butyrolactone small molecule inducers, which are discussed in more detail in section 1.5.4.1.

1.5.3 MICROCIN PRODUCTION BY *Escherichia coli*

Microcins are a family of low molecular weight antibiotic substances produced by various members of the Enterobacteriaceae. As a family they are related to bacteriocins, but are distinguished by their small size (< 10 kDa) and the fact that they are not inducible by the SOS response, but are produced as classical secondary

metabolites in the idiophase of the growth cycle. The best studied members of these peptide antibiotics are microcin B17 and microcin C7.

In *E.coli* the genes responsible for the production of microcin B17 (*mcb* genes) and microcin C7 (*mcc* genes) are organised as operons and are transcribed as monocistronic messages, *mcbA-G* and *mcc α - γ* respectively. Within these operons are the microcin structural genes which direct a complex series of post-translational modifications and genes for secretion and immunity (reviewed by Kolter and Moreno, 1992 and Moreno *et al.*, 1995). Microcin B17s mode of action is to block DNA replication by interacting with DNA gyrase trapping an abortive, cleavable, gyrase-DNA complex. The formation of this complex in the absence of an immunity mechanism is irreversible and ultimately leads to DNA degradation and cell death. Less is known about the mode of action of microcin C7 except that it is distinct from microcin B17 and that it blocks protein synthesis.

1.5.3.1 REGULATION OF MICROCIN B17 AND MICROCIN C7

Both microcin B17 and microcin C7 are known to be regulated at the transcriptional level in a growth phase dependent manner. However, the regulatory mechanisms for the production of these two microcins are slightly different. OmpR is known to activate the major promoter of the microcin B17 operon P_{mcb} . This is not the case for microcin C7 production. OmpR is the DNA binding response regulator, which is part of a two component system with protein EnvZ (the sensor histidine protein kinase component). Two component systems are known to transduce environmental signals. This signal activates the sensor kinase which in turn activates the response regulator protein in the system. The activation results from the autophosphorylation of the sensor kinase, which then transfers this phosphate to the response regulator (see Stock, 1989). The activated response regulator can then promote the transcription of genes usually specific to the environmental stimuli that activated it.

The primary role of OmpR is to regulate the expression of *ompC* and *ompF*, responsible for the production of outer membrane porins (reviewed by Dorman, 1994). OmpR is phosphorylated by EnvZ, in response to changes in osmolarity. Activated OmpR (OmpR ~ P) is then able to bind to specific sites within the promoter region of genes within its regulon, promoting the transcription of these genes by interacting directly with the α subunit of RNA polymerase (Slauch *et al.*, 1991).

Activation by OmpR is not the only mechanism for growth phase induction of microcin B17. The major promoter of this operon (P_{mcb}) is characteristic of a class of promoters called 'gearbox promoters' (Aldea *et al.*, 1990). Gearbox promoters are growth-phase sensitive, having -35 regions which share homology to the *E.coli* promoter consensus, but possessing differing -10 regions (Vicente *et al.*, 1991). Deletion of this -10 motif disrupts the growth phase control (Aldea *et al.*, 1990). Promoter fusion studies have shown that the expression from a gearbox promoter is inversely proportional to growth rate, with the maximal activity in stationary phase when growth rate is at its lowest. The exact mechanism by which transcription is regulated is unknown and may involve an alternative sigma factor. Vincente *et al.*, (1991) have put forward a model to explain the phenomenon of gearbox promoters. The model relies both on the affinity of these promoters for RNA polymerase being higher than that of other promoter types and that they are recognised by an alternative sigma factor which itself is regulated by a gearbox promoter. As cells enter stationary phase, promoters compete for a decreasing number of RNA polymerase molecules. The model of Vincente *et al.*, predicts that the high affinity gearbox promoters will, by their very nature, bind more RNA polymerase molecules leading to an increase in expression during this phase of growth. This mechanism is mediated partly by the higher concentration of the alternative sigma factor and a decline in the major *E.coli* sigma factor, σ^{70} .

It has been suggested that the *E.coli* stationary phase sigma factor encoded by the *rpoS* gene (σ^s) is responsible for stimulating the gearbox promoter found upstream of

the *ftsQ* gene (involved in cell division; Sitnikov *et al.*, 1996). The expression of P_{mcb} is virtually unaffected in *rpoS* mutants however, although sigma factor σ^s is known to regulate the idiophase expression of microcin C7 (Kolter and Moreno, 1992; Moreno *et al.*, 1995). This sigma factor is thought to regulate a number of genes required for protection and integrity of the cell during stationary phase. As with OmpR/EnvZ, the signal that induces the transcription of this sigma factor, other than a general depletion of nutrients, is unclear, although regulation by ppGpp has been implicated, linking this system of regulation to the stringent response (McCann *et al.*, 1993; Gentry *et al.*, 1993).

Other regulatory influences on the production of these microcins in *E. coli*, include MprA, recently characterised as a negatively acting regulatory protein. Deletion of the *mprA* gene results in a significantly increased level of transcription of both the microcin B17 and the microcin C7 operons (Del Castillo, 1991). The MprA protein shows a low level of homology with a number of bacterial regulatory proteins such as PecS and MarR (Reverchon *et al.*, 1994; Hachler *et al.*, 1991) and like them this protein has no obvious DNA binding motif and its exact mode of action is unclear. Del Castillo, (1991) suggests that it may act by altering DNA topology in a manner similar to the histone-like *E. coli* proteins (for reviews see Kolter and Moreno, 1992; Drlica and Rouviere-Yaniv, 1987).

1.5.3.2 REGULATION OF COLV IS DEPENDENT ON IRON CONCENTRATION

Another regulatory mechanism for the production of these peptide antibiotics is demonstrated by the microcin ColV. ColV is only synthesised when the concentration of iron in the media is low (Moreno *et al.*, 1995). The expression of ColV is mediated by the Fur iron repressor protein. In the presence of high concentrations of molecular iron Fur can bind DNA and repress transcription of genes with a characteristic binding motif. This type of regulation is not restricted to antibiotics as several siderophores are also under its control (Del Lorenzo *et al.*, 1988; Williams and Roberts, 1989).

TABLE 1

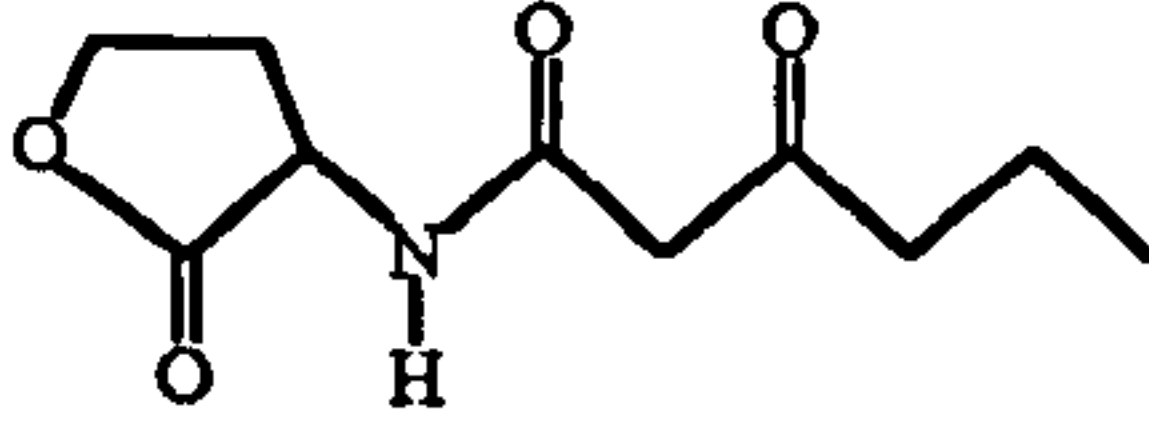
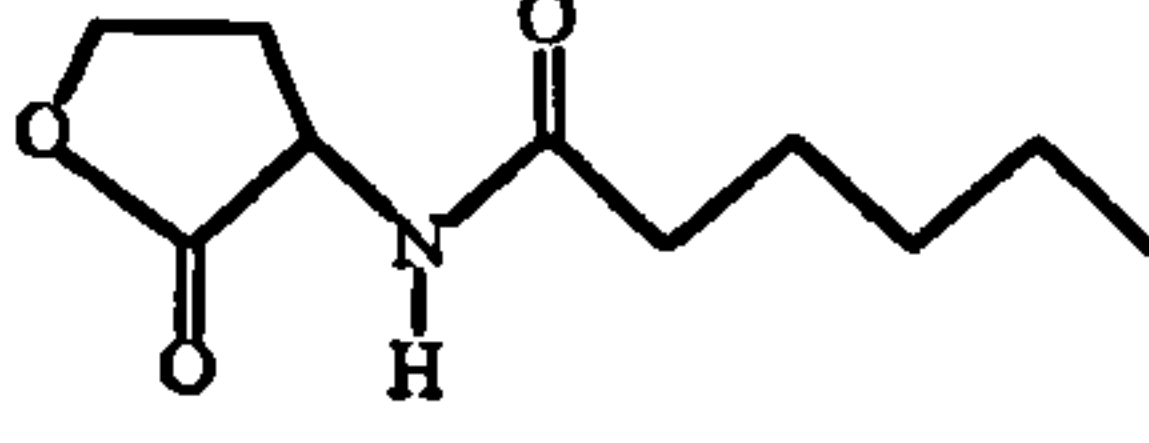
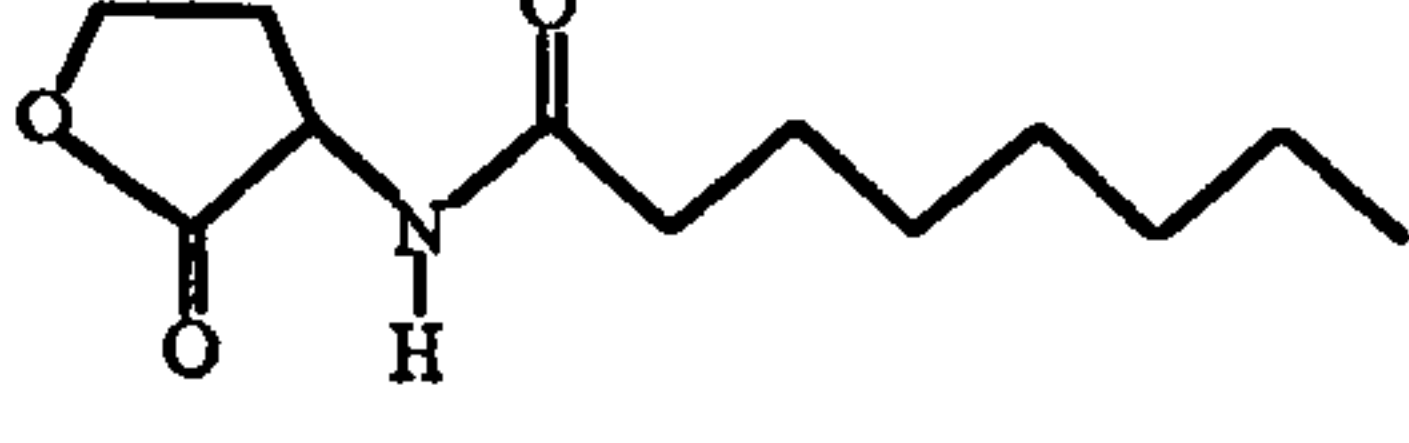
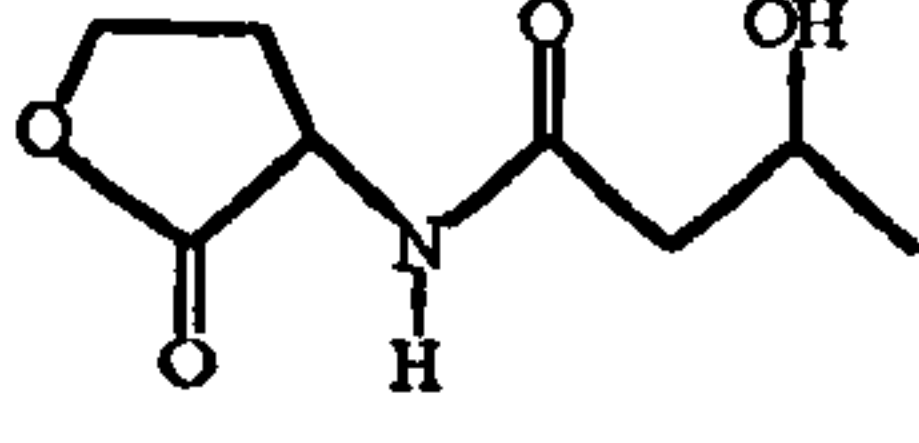
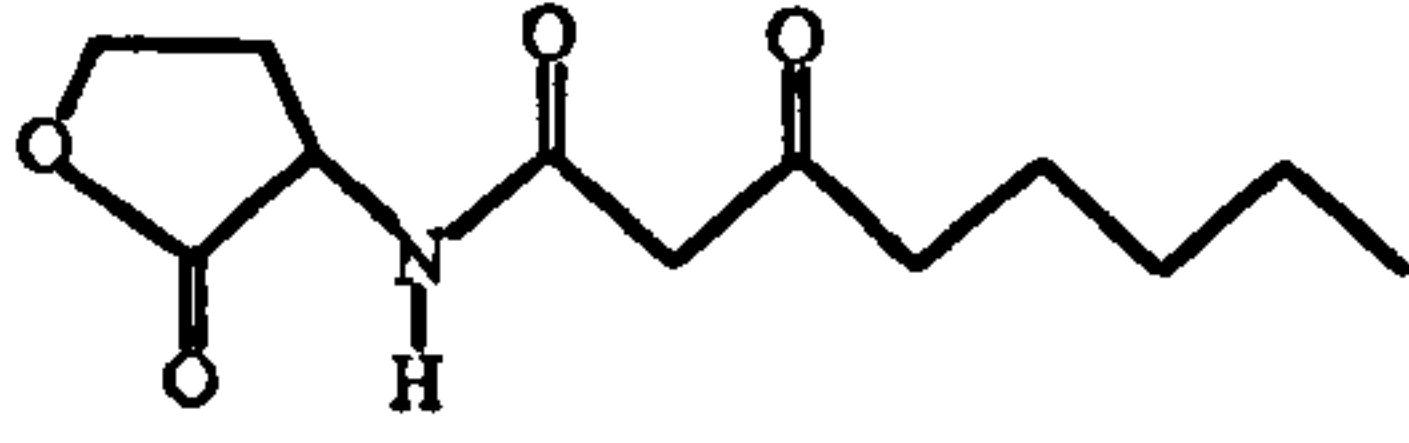
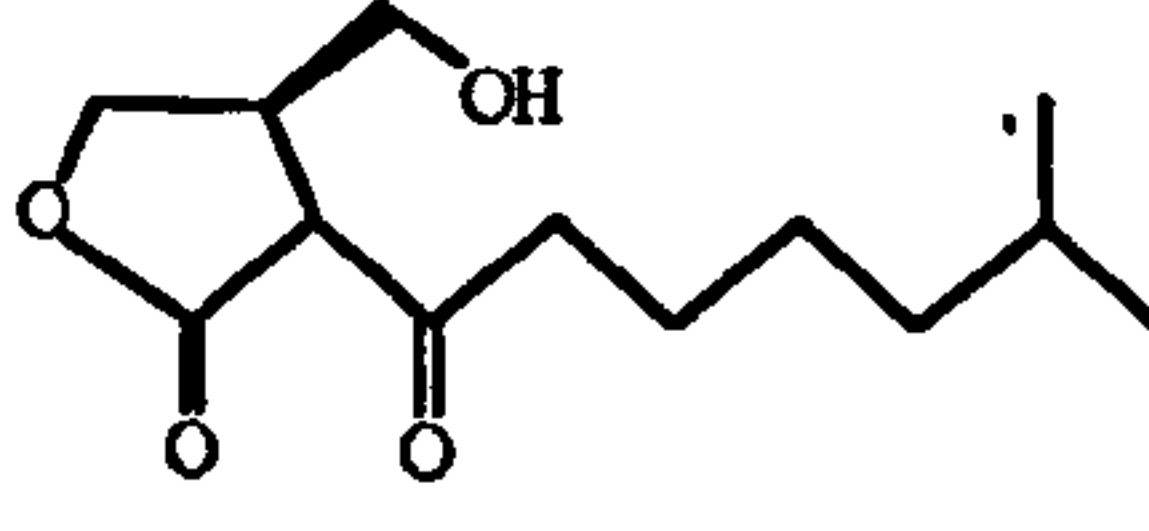
Pheromone		Organism	Phenotype
OHHL		<i>Photobacterium fischeri</i> AI-1	Bioluminescence.
		<i>Erwinia carotovora</i>	Carbapenem, Exoenzymes.
		<i>Pseudomonas aeruginosa</i>	Exoenzymes, Pigment
HHL		<i>Photobacterium fischeri</i> , AI-3	unknown
OHL		<i>Photobacterium fischeri</i> AI-2	unknown
HBHL		<i>Vibrio harveyi</i>	Bioluminescence
OOHL		<i>Agrobacterium tumefaciens</i>	Enhances conjugal transfer of Ti plasmid
A-factor		<i>Streptomyces griseus</i>	Streptomycin, Sporulation

Table 1 Examples of pheromones produced by a number of bacteria and the phenotypes they are purported to control. OHHL N-(3-oxohexanoyl)-L-homoserine lactone;HHL N-hexanoyl-L-homoserine lactone; OHL N-octanoyl-L-homoserine lactone;HBHL N-(3-hydroxybutanoyl)-L-homoserine lactone; OOHL N-(3-oxooctanoyl)-L-homoserine lactone.

1.5.4 THE BACTERIAL PHEROMONE: BIOLUMINESCENCE IN THE MARINE BACTERIUM *Photobacterium fischeri*, A PARADIGM FOR CELL DENSITY SENSING

Quorum sensing is a term which has been used to describe a system by which bacteria are able to monitor their own population density (Fuqua *et al.*, 1994). The bacteria produce a diffusible compound known as a bacterial pheromone which accumulates in the surrounding environment during growth. At low cell densities the concentration of this pheromone is low. As cell density increases so does the surrounding concentration of pheromone and at a particular threshold concentration the genes which require it for their activation are induced. The *lux* system is considered the archetypal system for the cell density sensing paradigm. The *lux* system directs the production of light, which does not serve any essential role in primary metabolism and so in this instance the production of the enzyme luciferase (required for bioluminescence) can be considered as a secondary metabolite, in keeping with the theme of this section.

Photobacterium fischeri is a marine bacterium found either free-living or in a symbiotic relationship in the light organs of certain deep sea fish (Dunlap and Greenberg, 1991). As previously mentioned the ability to sense cell density centres on the production of a highly diffusible small molecule known as a pheromone or, in this case, autoinducer (AI). In this instance it is conventional to call the *P.fischeri* pheromone an autoinducer because, in addition to bioluminescence, it also promotes its own synthesis (see later). The AI molecule regulating bioluminescence in *P.fischeri* was first described by Eberhard *et al.*, (1981) and is *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL; table 1).

The *lux* regulon can be split into two operons (figure 1.3), one containing *luxR* and the other *luxICDABEG* separated by an operator region. The genes *luxCDABEG* are responsible for providing the substrate and encoding the enzyme luciferase, for the production of light (Meighen, 1991). The product of *luxI* is proposed to be the

FIGURE 1.3

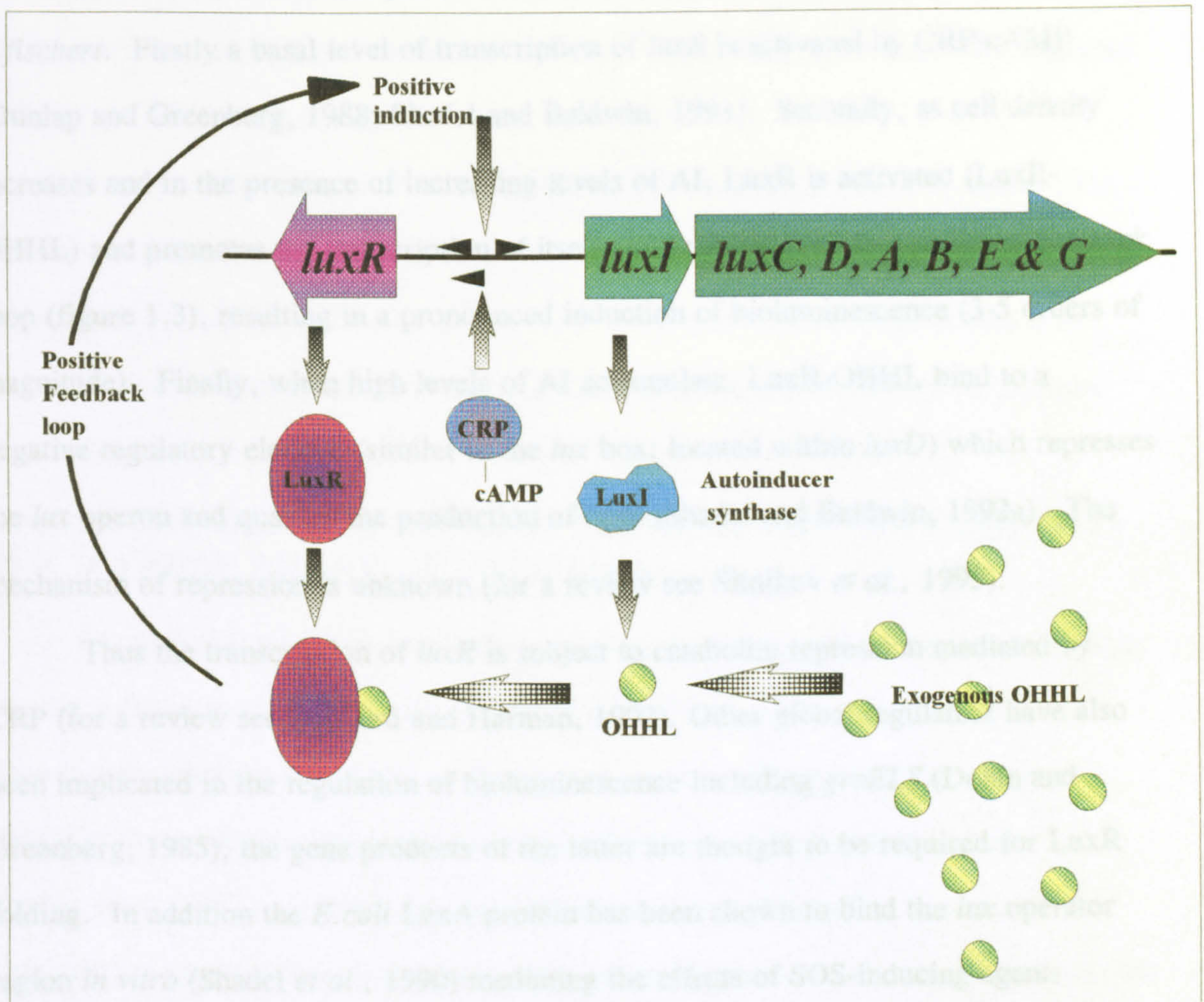


Figure 1.3 The bacterial pheromone: The regulation of bioluminescence in *Photobacterium fischeri*. The gene product of *luxR* is a receptor for, and is activated by, *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL). Activated LuxR promotes the transcription of itself, *luxI* (autoinducer synthase) and the genes required for bioluminescence in a positive feedback loop. The initial basal level of LuxR is provided by transcriptional activation by cAMP receptor protein (CRP; see text).

autoinducer synthase, required for the endogenous production of AI, and *luxR* is the transcriptional activator thought to be activated by an interaction with OHHL. It has been suggested that the activated LuxR binds to a 20bp inverted repeat (*lux* box) in the operator region of the *lux* regulon, thereby inducing the expression of *lux* (Devine, 1988; Stevens *et al.*, 1994).

There are three distinct phases to the induction of bioluminescence by *P.fischeri*. Firstly a basal level of transcription of *luxR* is activated by CRP-cAMP (Dunlap and Greenberg, 1988; Shadel and Baldwin, 1991). Secondly, as cell density increases and in the presence of increasing levels of AI, LuxR is activated (LuxR-OHHL) and promotes the transcription of itself and *luxICDABEG* in a positive feed back loop (figure 1.3), resulting in a pronounced induction of bioluminescence (3-5 orders of magnitude). Finally, when high levels of AI accumulate, LuxR-OHHL bind to a negative regulatory element (similar to the *lux* box; located within *luxD*) which represses the *lux* operon and quashes the production of light (Shadel and Baldwin, 1992a). The mechanism of repression is unknown (for a review see Sitnikov *et al.*, 1995).

Thus the transcription of *luxR* is subject to catabolite repression mediated by CRP (for a review see Botsford and Harman, 1992). Other global regulators have also been implicated in the regulation of bioluminescence including *groELS* (Dolan and Greenberg, 1985), the gene products of the latter are thought to be required for LuxR folding. In addition the *E.coli* LexA protein has been shown to bind the *lux* operator region *in vitro* (Shadel *et al.*, 1990) mediating the effects of SOS-inducing agents.

The LuxR protein is a 250 amino acid modular protein. Its C-terminus shows sequence similarity with many DNA binding proteins such as those involved in two component systems (Stock *et al.*, 1989), owing to its helix-turn helix DNA binding motif (Sitnikov *et al.*, 1995). Currently, although there is no direct evidence, it is thought that the N-terminus of LuxR interacts directly with OHHL (Shadel *et al.*, 1990; Sitnikov *et al.*, 1995). This interaction then, perhaps by some conformational change in the protein, allows the C-terminus of LuxR to bind the *lux* operator region.

Much less is known about the production of the AI molecule by LuxI (autoinducer synthase). The apparent situation in *P.fischeri* is even more complex than first anticipated because two more AI molecules are produced by this bacterium: *N*-octanoyl-L-homoserine lactone and *N*-hexanoyl-L-homoserine lactone, the former of these two does not rely on LuxI for its production (table 1; Dunlap and Kuo, 1992; Kuo *et al.*, 1994). LuxI was identified as an autoinducer synthase because bioluminescence can be reconstituted in *E.coli* transformed with *luxRCDABEG* with either the exogenous addition of OHHL or *luxI* added *in trans*.

One particular enigma concerning this type of regulatory system is that even in systems involving identical inducer molecules, such as *lux* and the regulation of carbapenem production in *Erwinia carotovora* subspecies *carotovora* (Chhabra *et al.*, 1992), the autoinducer synthases (LuxI and CarI) share only low identity (in this case only 25%).

1.5.4.1 CELL DENSITY SENSING SYSTEMS ARE REPRESENTED IN A DIVERSE ARRAY OF BACTERIA:

Quorum sensing is not restricted to esoteric marine bacteria. Analogous systems have been found to regulate a wide variety of phenotypes, such as the production of secondary metabolites and virulence factors in a number of bacteria. Table 1 shows several of these bacterial pheromones and the phenotypes they regulate (where known; Bainton *et al.*, 1992). Perhaps the most important group of bacteria, in terms of the production of commercially important secondary metabolites, are the streptomycetes. In *Streptomyces griseus*, A- factor (2-[6-methylheptanoyl]-3*R*-hydroxymethyl-4-butanolide; table 1) has been found to regulate sporulation, the production of a diffusible yellow pigment and the production of, and resistance to, streptomycin (Horinouchi and Beppu, 1994).

Although A-factor is related structurally to OHHL, its mode of action is different. Horinouchi and Beppu, (1994; summarised in figure 1.4) have put forward a model for the regulation of streptomycin by A-factor in which, at a certain threshold

FIGURE 1.4

Adapted from Horinouchi and Beppu, (1994)

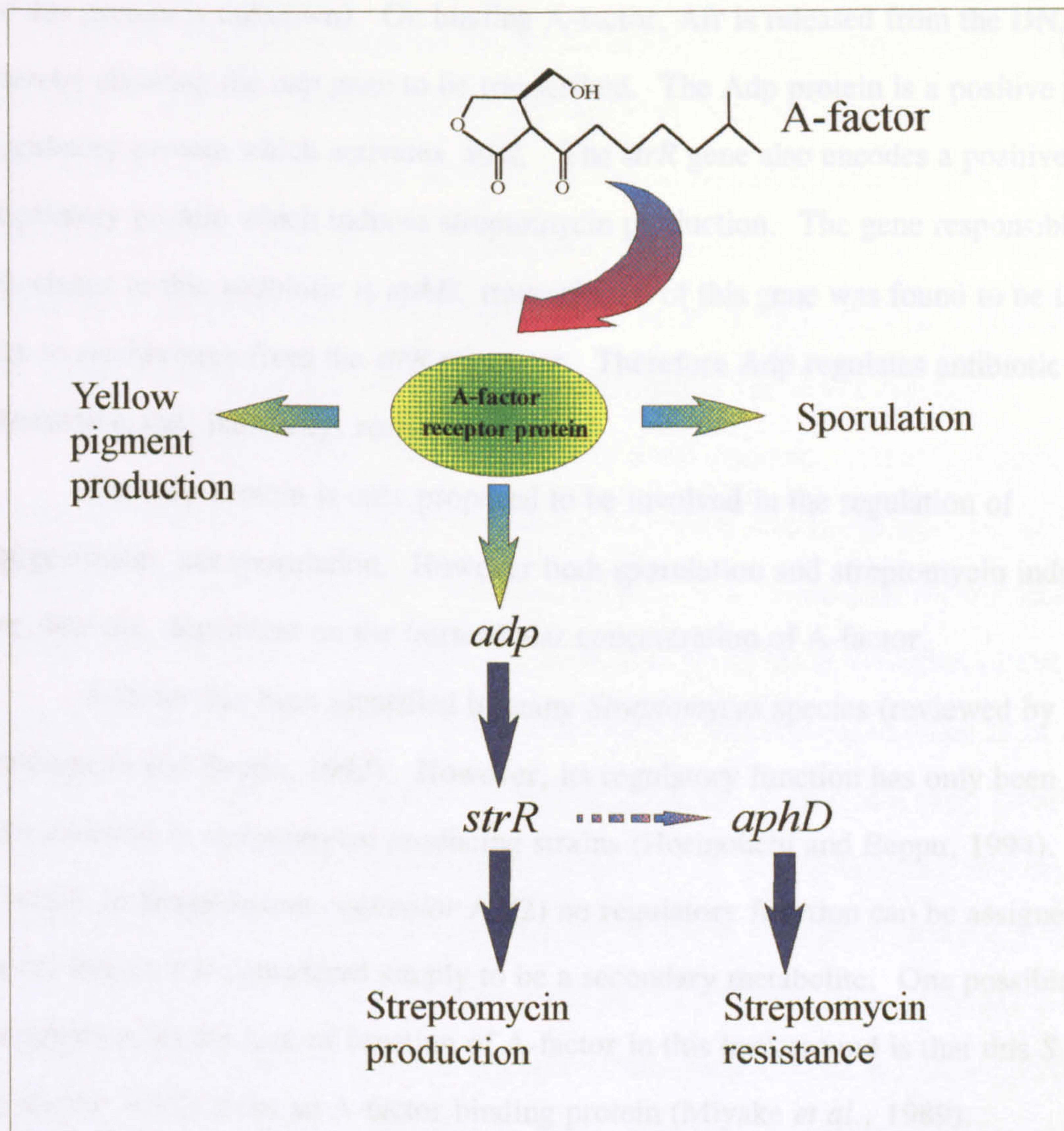


Figure 1.4 A model summarising the proposed interactions in the A-Factor regulon. A-Factor interacts with the A-Factor receptor protein (curved arrow), resulting in it being released from the promoter regions of various genes (most are undefined) thereby derepressing them (light coloured arrows). The gene *adp* (A-factor dependent protein) is proposed to be one of the genes derepressed by A-factor binding. The gene product of *adp* activates the transcription of *strR* resulting in streptomycin production, in addition, by readthrough from the *strR* promoter (broken arrow), it indirectly activates the transcription of *aphD*, the gene product of which confers streptomycin resistance. (Dark arrows indicate activation)

concentration of A-factor, it is bound by an A-factor receptor protein (Afr) which, unlike LuxR, is a repressor (Miyake *et al.*, 1990). In the absence of A-factor, Afr binds to DNA and by doing so is thought to repress the transcription of a number of genes, including a gene encoding a protein called A-factor-dependent protein (Adp; the nature of this protein is unknown). On binding A-factor, Afr is released from the DNA thereby allowing the *adp* gene to be transcribed. The Adp protein is a positive acting regulatory protein which activates *strR*. The *strR* gene also encodes a positive acting regulatory protein which induces streptomycin production. The gene responsible for resistance to this antibiotic is *aphD*, transcription of this gene was found to be largely due to readthrough from the *strR* promoter. Therefore Adp regulates antibiotic production and, indirectly, resistance.

The Adp protein is only proposed to be involved in the regulation of streptomycin, not sporulation. However both sporulation and streptomycin induction are, like *lux*, dependent on the intracellular concentration of A-factor.

A-factor has been identified in many *Streptomyces* species (reviewed by Horinouchi and Beppu, 1992). However, its regulatory function has only been characterised in streptomycin producing strains (Horinouchi and Beppu, 1994). For example in *Streptomyces coelicolor* A3(2) no regulatory function can be assigned to A-factor and so it is considered simply to be a secondary metabolite. One possible explanation for the lack of function of A-factor in this background is that this *S. coelicolor* A3(2) lacks an A-factor binding protein (Miyake *et al.*, 1989).

The following sections will now focus on the main theme of this project the production of antibiotic and pigment by *S.marcescens*. In the past a great deal of research has looked various pigments and antibiotics produced by bacteria. Each of the following sections will begin with a broad view of research relating to bacterial antibiotics and pigments and then will then focus in on research related more specifically to what is known about their production in *S.marcescens*.

1.6 BACTERIAL PIGMENTS

Many procaryotes produce pigments (Feistner,1990). The colour and proposed roles of these pigments are just as diverse as the organisms producing them. All iron complexes are coloured, pyoverdine is a yellow green siderophore produced by *Pseudomonas aeruginosa* required for the chelation of iron (Feistner,1990). Some pigments, such as the carotenoids, are thought to protect the bacterium from the harmful effects caused by irradiation by visible light and others act to harness light for the production of ATP, such as those produced by cyanobacteria. Many pigments show antibacterial activity, examples of which would be the blue pigment actinorhodin and the red pigment undecylprodigiosin made by *Streptomyces coelicolor* A3(2).

As mentioned earlier, the production of a red pigment: 2-methyl-3-pentyl-6-methoxyprodigiosin (or prodigiosin; figure 1.5A) is the phenotype most commonly associated with *Serratia* species. The development of a simple photometric test for prodigiosin has considerably increased our knowledge of the distribution of this type of pigment amongst procaryotes. The test relies on the fact that prodigiosin is an acid indicator which appears red/pink in an acid solution and yellow in a basic solution. A more definitive test exploiting the optical properties of this pigment has also been used (Williams and Quadri, 1980). Prodigiosin has different absorption maxima in acid and alkali solutions which is highly characteristic of this pigment; in isopropanol the maxima are 540 nm when acidified and 466 nm when made basic (MERCK, 1983). Prodigiosin production is not unique to the genus *Serratia*. Prodigiosin and prodigiosin related pigments have been identified in many bacteria such as *Pseudomonas magnesorubra*, *Vibrio psychroerythreus* and an increasing number of actinomycetes (Gerber and Lechevalier, 1976). Prodigiosin like pigments (referred to as Prodiginines) are pigments with the same basic chemical structure as prodigiosin but which have complex side chains and modifications. An example of a prodiginine is the pink pigment butylcycloheptylprodiginine produced by *Streptomyces coelicolor* A3(2) (figure 1.5B).

FIGURE 1.5

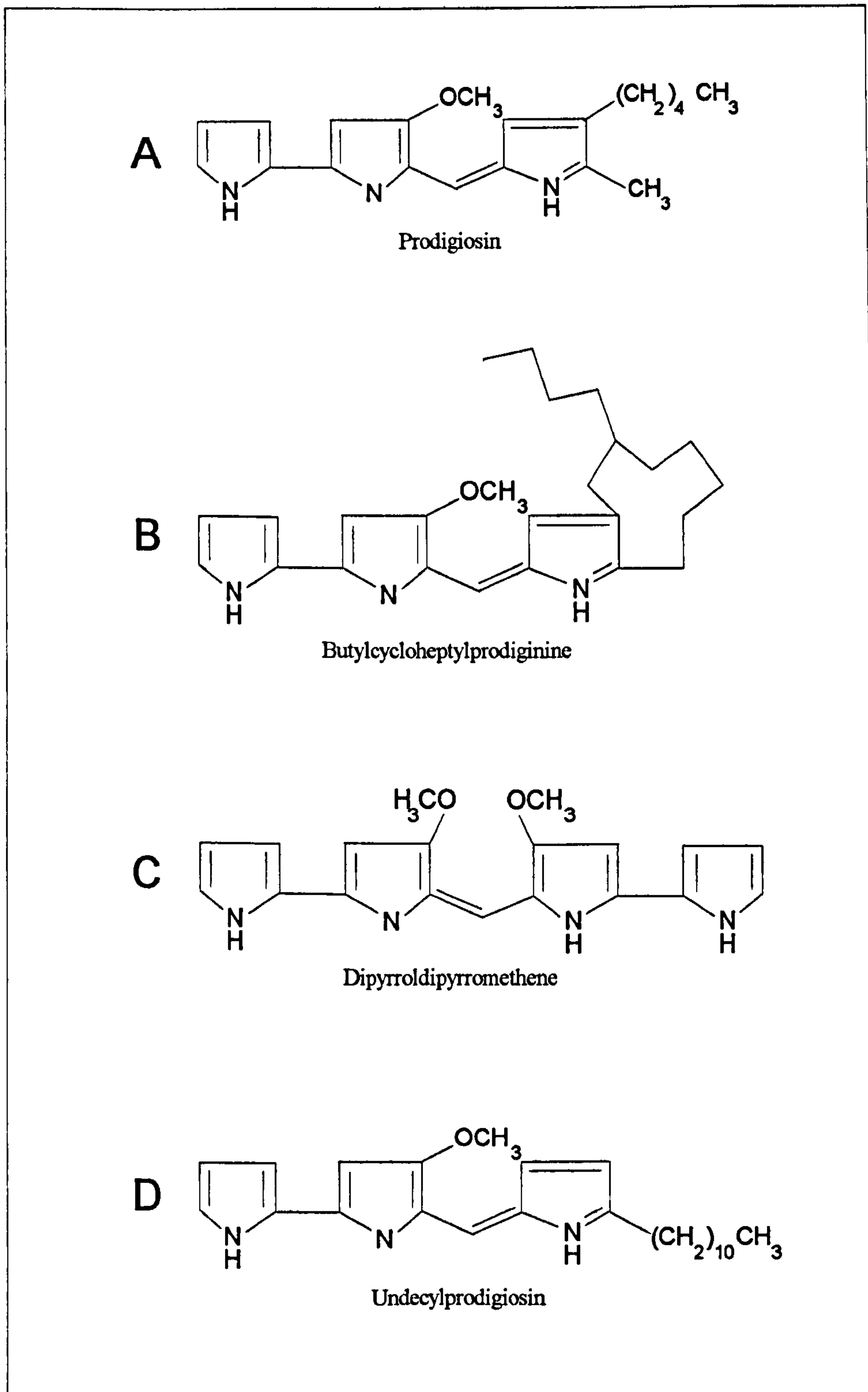


Figure 1.5 Examples of the structure of prodigiosin and prodigiosin-related pigments

1.6.1 PRODIGIOSIN AND THE GENUS *Serratia*

The ability to produce prodigiosin by *Serratia* species, perhaps surprisingly, is restricted to only three members of the genus: *Serratia marcescens*, *Serratia rubidae* and *Serratia plymuthica* (Grimont and Grimont, 1984). In addition there is a significant variation in the ability to produce pigment between *Serratia* biotypes. An enigma concerning pigment production by members of this genus, is that the majority of clinical strains of *Serratia marcescens* are non-pigmented (Ding, 1983) and a far higher proportion of environmental isolates produce prodigiosin. Grimont and Grimont, (1978) isolated various environmental strains of *Serratia marcescens* and reported that 22 biotypes out of 29 isolated were pigmented. One explanation for this is that, in the host, prodigiosin is a burden on the cell creating a selective disadvantage. However, Williams *et al.*, (1971) have shown that the growth kinetics of a pigmented strain of *S. marcescens* was identical to a non-pigmented strain, as measured by viable counts, total protein and oxygen uptake. Another idea mooted is that when clinical isolates are grown for identification they are not cultured for a sufficient period of time for pigment to appear (Grimont, 1978). This theory is not supported by the findings of Ding and Williams, (1983) however, who investigated whether clinical isolates could produce prodigiosin if they were furnished with biosynthetic intermediates (see section 1.6.6). A total of 65 white *S.marcescens* strains were tested; 14 of these strains could not produce pigment, 48 strains produced an unidentified grey or pink pigment and only 3 had the ability to make prodigiosin and only then when furnished with a series of intermediates.

1.6.2 POSSIBLE FUNCTIONS ATTRIBUTABLE TO PRODIGIOSIN

There have been many attempts to assign a function to prodigiosin. Paruchuri and Harshey, (1978) have reported that colour variation is associated with a change in surface antigens, which may help evade the host immune system. However this conclusion is at odds with the observation that the majority of clinical isolates are non-pigmented. It has been suggested that the majority of environmental isolates are

pigmented because the hydrophobic nature of this pigment aids the dispersal of this bacterium and adherence to oils, sediments and non wettable plastics allowing the colonisation of various environmental niches and thereby creating a selective advantage (Burger, 1985; Rosenberg, 1984). Another possibility is that, because water-insoluble organic matter constitutes the major source of soil nutrients (Demain, 1995), the hydrophobic pigment may aid the search for these nutrients. However, Rosenberg, (1984) using an enrichment technique to test the ability of *S.marcescens* pigment variants to bind to hydrocarbons and polystyrene, found that hydrophobicity was not solely due to prodigiosin and that there were several other unidentified factors contributing to bacterial adherence which were common to both pigmented and non pigmented strains. This is consistent with findings of Matsuyama *et al.*, (1986) where Serrawettin, which is found in vesicles closely associated with prodigiosin, was concluded to modify cell surface hydrophobicity. Other functions put forward are based on the antimicrobial activity of prodigiosin and the fact that it is a potent photosensitiser which may be lethal to other bacteria when released into the environment (Williams and Quadri, 1980).

1.6.3 THE PROPERTIES OF PRODIGIOSIN

Prodigiosin has various properties considered of clinical importance, including antimalarial, antifungal and antiprotozoal activities (Williams and Quadri, 1980). Prodigiosin is more active against Gram positive bacteria than Gram negative, although Cox, (1995) reported that prodigiosin produced a significant zone of antibiosis on an *Escherichia coli* strain ESS bioassay. Amoebae that engulf pigmented *S.marcescens* cells are reported to either encyst or die, whereas no such effect is observed for non-pigmented strains (Demain, 1995). In addition prodigiosin 25C, produced by *Streptomyces hiroshimensis*, was found to be a potent immuno-suppressant in mice affecting primarily T-cell poliferation (Nakamura *et al.*, 1986). This property, in mice

at least, may be of clinical importance for the treatment of Graft versus Host reactions (Ryohei *et al.*, 1992)

1.6.4 EXTRINSIC FACTORS CONTROLLING PRODIGIOSIN PRODUCTION

Prodigiosin is a classical secondary metabolite; it has no obvious function, prodigiosin null mutants are not affected in their viability and its production begins in the transition from trophophase to idiophase. In liquid cultures of *S.marcescens*, pigment becomes visible after 8-12 hours reaching a peak after 24-48 hours (Williams *et al.*, 1971). When culturing *S.marcescens* in the laboratory it becomes obvious that many conditions, such as media composition and temperature, affect the production of pigment (reviewed by Williams and Quadri, 1980). Heightened levels of pigment production can be observed on minimal or peptone glycerol media compared to rich nutrient media. Because growth rate has a strong influence on prodigiosin biosynthesis, and because factors affecting pigment production could not be measured quantitatively, researchers developed a system named “Non-proliferating cells” (NPCs) to separate cellular proliferation from pigment production. NPCs were first described by Williams *et al.*, (1971) in a chance observation that cultures grown at 38°C (at this temperature *S.marcescens* is non pigmented) could, if shifted to 27°C, form pigment when supplied with certain amino acids. To make NPCs *S.marcescens* cells are grown for 72 hours at 38°C and then washed and suspended in saline at 27°C. Following induction of pigmentation using proline, factors which affect this induction can then be studied without interference from growth rate/phase (Williams *et al.*, 1971).

When the effect of temperature has been studied on actively growing cells and NPCs, prodigiosin is seen to be synthesised between the relatively narrow margins of 12-36°C whereas growth is supported between 4-42°C. Pigment production by NPCs is slightly different with no pigment produced below 13°C or above 32°C (Williams *et al.*, 1971). These effects of temperature are thought to be caused, at least in part, by the sensitivity to elevated temperatures of enzymes in the known stages of the pathway

(Williams and Quadri, 1980). Williams *et al.*, (1971) attempted to study the effect of pH on pigment production by NPCs. However prodigiosin has an intrinsic buffering property (Ruis *et al.*, 1994). No matter what the initial pH of the medium invariably the final pH, unless the media itself is buffered, will be between pH 7.2 and 8.0 (Williams and Quadri, 1980). In a recent study by Sole *et al.*, (1994) using buffered media, prodigiosin was found to be produced by NPCs at between pH 5.5 and 9.5, with maximal production being between pH 8.0-8.5.

As previously mentioned, media composition can have a profound effect on pigment production. NPCs grown in minimal media required casein hydrolysate for the induction of pigment (Williams *et al.*, 1971). This observation has led to many experiments investigating the induction of pigment by certain amino acids. Only those amino acids that could be used as carbon or nitrogen sources were found to induce pigmentation, i.e. the amino acids must be metabolised by NPCs prior to initiating prodigiosin biosynthesis (Williams and Quadri, 1980). This implies, as you may expect, that any factor that affects primary metabolism will also affect the production of this secondary metabolite, calling into question whether the results obtained using NPCs relate directly to the regulation of prodigiosin biosynthesis or just a 'knock on' effect from primarily metabolism.

1.6.5 GLOBAL CONTROL OF PRODIGIOSIN PRODUCTION

There have been few genetic or biochemical studies on prodigiosin production by *S.marcescens*. However, there have been several anecdotal reports of common regulatory links between prodigiosin production and several other phenotypes. Hines *et al.*, (1988) found when isolating mutants defective for the production of extracellular enzymes that there was a correlation between protease production and prodigiosin production, where 20% of the protease mutants were also non-pigmented. Consistent with this, Goluszko *et al.*, (1995) isolated *S. marcescens* transposon mutants which were

FIGURE 1.6

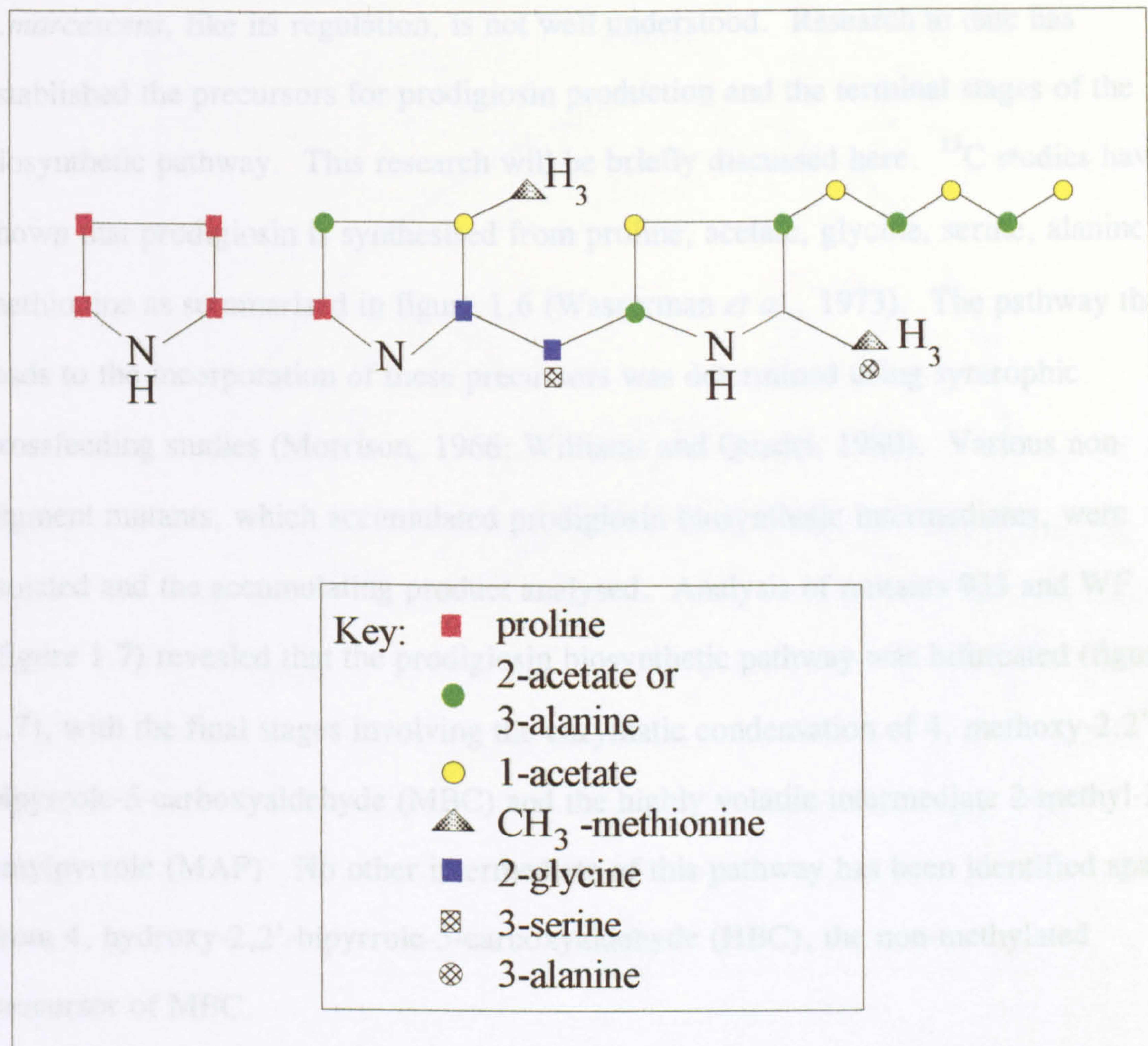


Figure 1.6 Patterns of incorporation of biosynthetic precursors into prodigiosin. (taken from Williams and Quadri, 1980)

The numbers (or methyl group) preceding the ¹³C radio labelled substrates indicates the carbon atom that was found to be incorporated into the prodigiosin molecule. Proline was found to be incorporated intact.

deficient for the production of protease, prodigiosin and also haemagglutinating activity. The genetic explanation for these observations has yet to be ascertained

1.6.6 THE PRODIGIOSIN BIOSYNTHETIC PATHWAY

The biosynthetic pathway leading to the production of prodigiosin by *S.marcescens*, like its regulation, is not well understood. Research to date has established the precursors for prodigiosin production and the terminal stages of the biosynthetic pathway. This research will be briefly discussed here. ^{13}C studies have shown that prodigiosin is synthesised from proline, acetate, glycine, serine, alanine and methionine as summarised in figure 1.6 (Wasserman *et al.*, 1973). The pathway that leads to the incorporation of these precursors was determined using syntrophic crossfeeding studies (Morrison, 1966; Williams and Quadri, 1980). Various non-pigment mutants, which accumulated prodigiosin biosynthetic intermediates, were isolated and the accumulating product analysed. Analysis of mutants 933 and WF (figure 1.7) revealed that the prodigiosin biosynthetic pathway was bifurcated (figure 1.7), with the final stages involving the enzymatic condensation of 4, methoxy-2,2'-bipyrrole-5-carboxyaldehyde (MBC) and the highly volatile intermediate 2-methyl-3-amylypyrrole (MAP). No other intermediate of this pathway has been identified apart from 4, hydroxy-2,2'-bipyrrole-5-carboxyaldehyde (HBC), the non-methylated precursor of MBC.

Some of the mutants isolated in this putative pathway cannot produce prodigiosin but are still pigmented. Mutant OF, which is blocked at the stage after the synthesis of HBC, produces an orange pigment called norprodigiosin (2-methyl-3-pentyl-6-hydroxy prodigiosene) by the condensation of HBC and MAP (figure 1.7). This pigment is not produced by wild type strains. It is also possible to isolate mutants which produce a deep blue or purple coloured pigment called dipyrroldipyrromethene, thought to be formed by the condensation of two MBC moieties (figure 1.5C; Williams and Quadri, 1980).

FIGURE 1.7

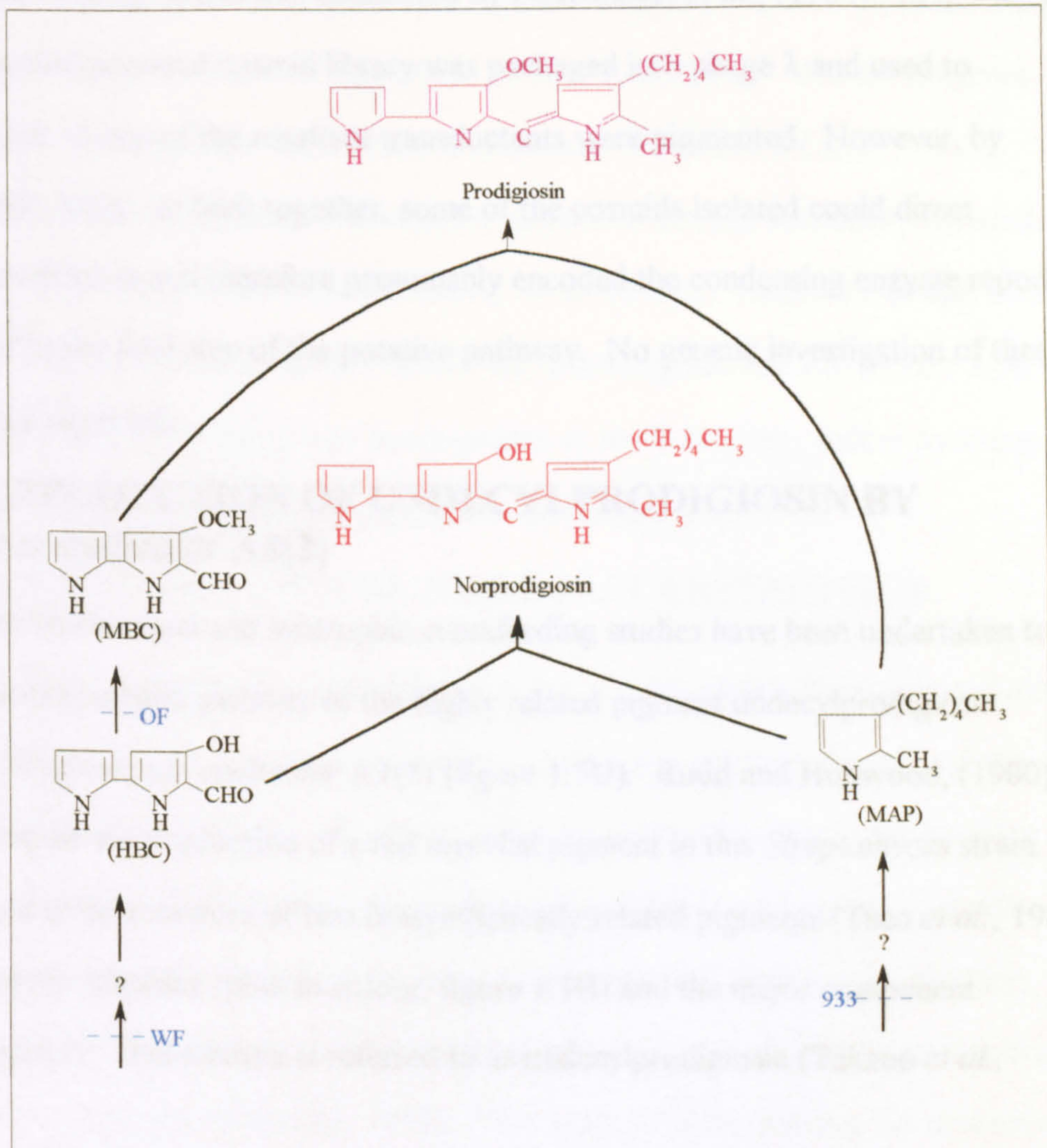


Figure 1.7 The proposed bifurcated pathway for prodigiosin biosynthesis in *Serratia marcescens*. (taken from Williams and Quadri, 1980). This pathway was developed by co-synthesis studies. Mutants WF, 933 and OF were instrumental in constructing this pathway (see text). The mutants are marked in blue indicating the position in the pathway at which they are blocked. Unknown intermediates are marked by a question mark. Norprodigiosin is produced only by mutants blocked in the production of MBC (such as OF), by the condensation of HBC and MAP. MBC 4-methoxy-2,2'-bipyrrol-5-carboxyaldehyde; HBC hydroxybipyrrole carboxyaldehyde; MAP methyl-3-amylpyrrole; Prodigiosin 2-methyl-3-pentyl-6-methoxyprodigiosin; Norprodigiosin 2-methyl-3-pentyl-6-hydroxyprodigiosin.

Little or nothing is known about the enzymes involved in prodigiosin biosynthesis and the genes encoding them, especially for the early stages of the pathway. The most recent research relating to this was conducted by Dauenhauer *et al.*, (1984). In this study a *S.marcescens* chromosomal cosmid library was packaged into phage λ and used to transduce *E.coli*. None of the resultant transductants were pigmented. However, by supplying MAP, MBC, or both together, some of the cosmids isolated could direct prodigiosin production and therefore presumably encoded the condensing enzyme reported to be involved in the final step of the putative pathway. No genetic investigation of these clones has been reported.

1.6.7 THE PRODUCTION OF UNDECYLPRODIGIOSIN BY *Streptomyces coelicolor* A3(2)

Similar biochemical and syntrophic crossfeeding studies have been undertaken to determine the biosynthetic pathway of the highly related pigment undecylprodigiosin produced by *Streptomyces coelicolor* A3(2) (figure 1.5D). Rudd and Hopwood, (1980) were first to report the production of a red mycelial pigment in this *Streptomyces* strain. It was later found to be a mixture of two biosynthetically related pigments (Tsao *et al.*, 1985); butylcycloheptylprodiginine (pink in colour; figure 1.5B) and the major component undecylprodigiosin. This mixture is referred to as undecylprodigiosin (Takano *et al.*, 1992).

Initial studies showed that *S.marcescens* could cross feed *S. coelicolor* A3(2) undecylprodigiosin mutants. This indicated that there was a certain degree of similarity between the pigment biosynthetic pathways of these two genera. Co-synthesis studies, between *S.marcescens* and *S. coelicolor* A3(2), were successful in isolating *S. coelicolor* A3(2) pigment mutants which resembled some of the *S.marcescens* mutants in the proposed bifurcated pathway (figure 1.7; Feitelson and Hopwood, 1983). This led to the eventual isolation of the genes encoding the whole pathway, cloned on a chromosomal fragment of 35.7 kb (Malpartida *et al.*, 1990). Using the various classes

of mutant, complementation analysis indicated that the pathway was composed of a minimum of 18 genes. The MAP branch of this pathway is thought to be more complex than that proposed for *Serratia* (figure 1.7), and this is thought to be consistent with the production of three pigments in *S. coelicolor* (Coco *et al.*, 1991). One of the genes sequenced in this cluster was *redD* which is discussed in section 1.5.2.1.

As previously mentioned *S. marcescens* produces another secondary metabolite (carbapenem) belonging to the β -lactam family of antibiotics. Because carbapenems are more recent additions to the β -lactam family the following section will briefly look at the history behind the discovery and development of the β -lactams, before focusing on more recent research relating to the production of carbapenems.

1.7 THE DISCOVERY OF THE β -LACTAM ANTIBIOTICS

In a lecture given by Sir Alexander Fleming, (1946) chemotherapy was defined as “*any treatment in which a chemical is administered in a manner directly injurious to the microbes infecting the body*”. Early examples of chemotherapeutic agents were essentially antiseptics: these various chemicals included boric acid, iodine and silver salts. They were administered directly to the wound and had little systemic utility, most, being unable to penetrate the tissue surrounding the wound, and so re-infection was a common occurrence (Fleming, 1946). The basis of administering the treatment was very empirical and in many cases the chemical was more toxic than the microbe itself.

In 1928 Fleming noticed that a mould (*Penicillium*) produced a diffusible antibacterial agent, which he called Penicillin (figure 1.8). The potential of Penicillin was not realised in the clinical environment until as late as 1940, when it was purified by Chain and Florey and shown to be a potent antibacterial agent, which was non toxic to animals and humans, and could be used to treat previously incurable illnesses.

Since the discovery of penicillin, the β -lactam family of antibiotics (of which penicillin is a member) has grown dramatically. All the subgroups are related by having

FIGURE 1.8

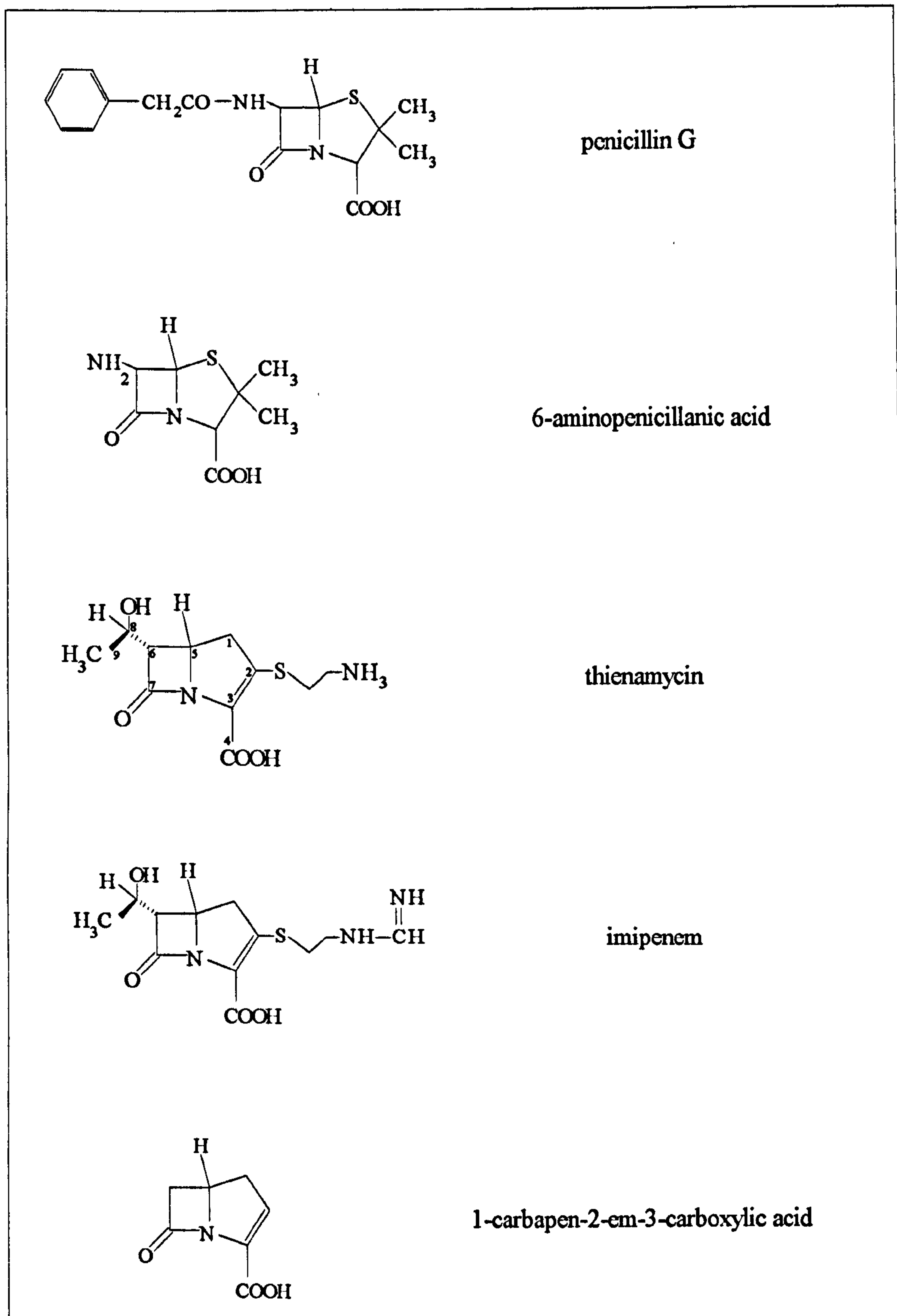


Figure 1.8 Examples of β -lactam antibiotics. The carbon atoms of thienamycin are numbered to show the differences between the penicillins and carbapenems (see text)

the same basic β -lactam ring structure (figure 1.8). This β -lactam family includes (in order of discovery): penicillins, cephalosporins, carbapenems, oxacephins and the monobactams (Rolinson, 1995). The development of penicillins for clinical use has led to some dramatic changes to its basic structure following the discovery of the basic penicillin nucleus, 6-aminopenicillanic acid (6-APA; figure 1.8), made naturally by *Penicillium chrysogenum* (Batchelor *et al.*, 1959). This simple β -lactam molecule has been used extensively for the synthesis of semi-synthetic antibiotics, such as ampicillin, whereby changes in the structure of the side chain have altered the spectrum of activity and overcome some problems associated with bacterial resistance.

β -lactam antibiotics are effective antimicrobial agents because they are structural analogues of the substrate of peptidoglycan transpeptidase (penicillin binding protein or PBP) involved in cross linking strands of peptidoglycan with the result that cell wall synthesis is disrupted. Resistance mechanisms employed by bacteria to β -lactams take three forms: the most common is the production of a β -lactamase enzyme which degrades the molecule by hydrolysing the β -lactam ring. The other mechanisms are altered PBPs, which no longer recognise β -lactam molecules, or reduced permeability of the outer membrane by the loss of membrane porins (Livermore, 1993).

The impetus for continued research into the development of new and novel antibiotics is obvious when you consider the emergence of multiply antibiotic resistant pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA) strains. Infections caused by strains such as this are recalcitrant to treatment using many therapeutically important antibiotics, which is of increasing concern (Archer and Niemeyer, 1994). The recently discovered carbapenem antibiotics are of potential clinical importance because they are resistant to many clinically encountered β -lactamases, as will be discussed below.

1.7.1 THE CARBAPENEMS

The first member of the carbapenems discovered was thienamycin made by *Streptomyces cattleya* (Kahan *et al.*, 1983). It was discovered in a systematic screening campaign in the 1970s initiated in response to the upsurge of bacterial antibiotic resistance.

To date over 40 naturally occurring carbapenems have been identified, most of which are made by *Streptomyces* species. Due to either low antimicrobial activity or a high level of chemical instability the majority of these have not been developed for clinical use (Wise, 1986). Thienamycin is a potent antimicrobial agent, but is also an unstable molecule (figure 1.8). This instability led to the development of imipenem, a N-formimidoyl derivative of thienamycin (figure 1.8; Leanza *et al.*, 1979), which has proven to be sufficiently stable to be used clinically and proving to be very effective in the treatment of most common community-acquired and nosocomial infections (Heu, 1994). Imipenem like thienamycin has a broad spectrum of activity against both Gram negative and Gram positive bacteria, aerobes, anaerobes, fermenters and non-fermenters (Heu, 1994) and is resistant to the majority of clinically encountered β -lactamases (Kahan *et al.*, 1983; Labia *et al.*, 1986). In clinical preparations imipenem is given as a combined treatment because even though it is resistant to bacterial degradation it is susceptible to human renal dehydropeptidase I. Therefore a combined preparation is used, called primaxin, which contains cilastatin an inhibitor of this enzyme (Heu, 1994). Even with this increased resistance to bacterial degradative enzymes there have been several reports of bacterial resistance. Strains of *Serratia marcescens*, *Enterobacter cloacae* and *Pseudomonas aeruginosa* have been isolated which are either partially or fully resistant to imipenem (Livermore, 1993). However, the enzymes for the resistance are not well represented amongst clinically important bacteria and there is no evidence that they are transmissible.

Studies investigating the biosynthesis of thienamycin have been hampered by the slow growth, poor genetic tractability of the producer organism, and low titres of production (Wise, 1986). In addition *Streptomyces cattleya* produces a number of other secondary metabolites (Williamson *et al.*, 1985). Genetic studies that have been reported suggest that carbapenems are produced by a biochemical pathway distinct from that of the penicillins and cephalosporins (Williamson *et al.*, 1985).

The problems associated with the production of thienamycin by *Streptomyces cattleya* have meant all the carbapenems used clinically today are made by total chemical synthesis in a 21 stage process being costly, inefficient and polluting. This high cost of production is balanced against a low dose regime (Hoffmann, 1993). Following the discovery of thienamycin two members of the Enterobacteriaceae were found to produce a carbapenem antibiotic (Parker *et al.*, 1982). These organisms (*Erwinia* and *Serratia*) have proven to be ideal genetic backgrounds for research into the production of carbapenem, as discussed below.

1.7.2 THE PRODUCTION OF CARBAPENEM BY *Serratia marcescens* AND *Erwinia carotovora* SUBSPECIES *carotovora*.

In a more recent screening campaign *S. marcescens* and *Erwinia carotovora* subspecies *carotovora* strain ATCC39048 (*Ecc*) were both found to produce the simple carbapenem antibiotic, 1-carbapen-2-em-3-carboxylic acid (referred to as carbapenem; figure 1.8; Parker *et al.*, 1982). The structure of carbapenem differs from that of penicillins by the lack of a sulphur moiety in the five membered thiazolidine ring and the side chain at position C6 does not have an amide bond. Biochemical studies have shown that the precursors of carbapenem are L-glutamate and acetate or malonate, which are also proposed as the precursors for thienamycin made by *Streptomyces cattleya* (Bycroft *et al.*, 1988; Williamson *et al.*, 1985). This differs considerably from those proposed for penicillins: L- α -aminoadipic acid, L-cysteine and L-valine (Neusch *et al.*, 1987), consistent with the idea that carbapenems are produced via a novel biosynthetic pathway (Williamson *et al.*, 1985).

At the beginning of this study there was very little known about the production of carbapenem by *S.marcescens*. Most research had focused on *Ecc*, genetic systems for which were well established. Because the carbapenem molecule made by *S.marcescens* was identical to that of *Ecc* it was anticipated that there would be a considerable degree of similarity between the two systems. Therefore the genes and regulation (discussed in the following section) of carbapenem in *Ecc* will be briefly discussed for comparisons to be drawn. Recently the putative *Ecc* carbapenem biosynthetic cluster has been cloned and sequenced (McGowan *et al.*, in press). From the sequence 8 open reading frames have been identified (*carA-H*), some of which share similarity to genes in a cluster involved in clavulanic acid synthesis (S.McGowan pers.comm). The cluster also encoded a protein (CarR) that was homologous to LuxR, the positive acting regulatory gene from *Photobacterium fischeri* (see section 1.5.4).

1.7.3 THE REGULATION OF CARBAPENEM: ANALOGOUS TO THE SYSTEM REGULATING BIOLUMINESCENCE IN *Photobacterium fischeri*

Research looking at the regulation of carbapenem in *Ecc* began with the isolation of a number of carbapenem non-producing mutants (Car^-) after chemical and transposon mutagenesis. Crossfeeding studies grouped these mutants into two classes (Bainton *et al.*, 1992a), Class 1 mutants were found to produce a low molecular weight highly diffusible molecule essential for the induction of carbapenem production by Class 2 mutants. This molecule was later identified as *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) identical in structure to that controlling bioluminescence in the marine bacterium *Photobacterium fischeri* (Bainton *et al.*, 1992b; see table 1).

Subsequent genetic analysis of both these classes of mutants revealed that Class 1 mutants had genetic lesions in a gene (*carR*; McGowan *et al.*, 1995) encoding a protein found to be homologous to the *P.fischeri* transcriptional activator LuxR. Class 2 mutants were defective in a gene (*carI*) similar to the autoinducer synthase encoded by the gene *luxI* (Swift *et al.*, 1993). *carI* mutants of *Ecc* were later found not only to be

affected in carbapenem production but also in the production of their exoenzyme virulence determinants (Jones *et al.*, 1993; Pirohnen *et al.*, 1993). The addition of exogenous OHHL or the *carI* gene *in trans* concomitantly restored both phenotypes, indicating that OHHL is responsible for the global regulation of both virulence and secondary metabolism in *Ecc*.

As with bioluminescence (section 1.5.4), the induction of carbapenem is also cell density dependent with antibiotic production being induced at a certain threshold concentration of OHHL. This induction is thought to involve the direct binding of DNA by the OHHL activated CarR protein, which then activates expression of the *car* operon. However, there are several notable differences between the *lux* and *car* systems. Firstly the *luxI* and *luxR* genes are linked to the luciferase biosynthetic genes (*luxCDABE*) whereas, although *carR* is linked to the carbapenem biosynthetic gene cluster (*carA-H*), *carI* is not (McGowan *et al.*, 1995). The genes *luxI* and *luxR* in *P.fischeri*, under non-inducing conditions, are expressed at low basal levels until the OHHL concentration reaches a threshold thought to be around 1ng/ml. However, the *Ecc carI* and *carR* genes are constitutively expressed throughout growth and carbapenem production is induced at the much higher threshold concentration of 0.5µg/ml of OHHL (Bainton *et al.*, 1992).

There are also differences in the production of carbapenem between *Serratia* and *Ecc*. There is no genetic or biochemical evidence for a small autoinducer molecule in *S.marcescens* strain ATCC39006 (G.P.C.Salmond pers comm; although another strain of *Serratia marcescens* has been found to produce OHHL-Bainton *et al.*, 1992).

Biochemical studies on the production of carbapenem in *S.marcescens* have found that it is produced in line with growth and not induced in late log phase as is found in *Ecc* (Bycroft *et al.*, 1988). As a direct result of this, *S. marcescens* produces significantly more carbapenem than *Ecc*. The fact that carbapenem is produced in parallel with growth in *S.marcescens* means that this antibiotic does not conform to the original definition of a secondary metabolite (Bu'Lock, 1961). However it does conform to the

definition given by Williams *et al.*, (1989b) by playing no role in the internal economy of the organism and so for the purposes of this study is considered to be a secondary metabolite.

The initial reasons for studying the regulation of carbapenem in *S.marcescens*, apart from the fact that it produced more than *Ecc*, were that, although *Serratia* and *Ecc* produce an identical carbapenem molecule, the regulatory mechanisms leading to the production of carbapenem must be significantly different between the two genera. In addition, work I carried out prior to starting this project (described in detail in section 3.0) indicated that there was a possible regulatory link between antibiotic and pigment production in *S.marcescens*. Pleiotropic mutants, generated by chemical mutagenesis, were isolated that were defective for both carbapenem and pigment production. From reversion studies it was clear that the genetic explanation for this centred on a single locus that was denoted Rap (Regulation of antibiotic and pigment). This was of special interest because, unlike carbapenem in this strain, prodigiosin is produced in the manner of classical secondary metabolites, in idiophase (as classified by Bu'Lock, 1961).

After starting this study Cox, (1995) sequenced the carbapenem biosynthetic cluster from *S. marcescens* and found that the genes shared a significant degree of homology with those of the *Ecc car* cluster (ranging from 65%-91% amino acid identity). In addition, *S.marcescens* also has a *luxR* homologue upstream of its biosynthetic cluster, which, unlike the *Ecc CarR* protein, is OHHL independent and so activates the *car* cluster constitutively. This is thought to explain why carbapenem is produced throughout the growth cycle (Cox, 1995).

The presence of a homologue of a cell density dependent transcriptional activator (CarR) could explain the proposed global regulatory link between antibiotic and pigment production in *S.marcescens*. Homologues of CarR have been shown to regulate multiple phenotypes. LasR from *Pseudomonas aeruginosa* has been shown to regulate alkaline protease and exotoxin A expression (Gambello and Inglewski, 1991; Gambello *et al.*, 1993). However, the work of Cox, (1995) has shown that *carR* in multicopy could not

complement pleiotropic *S.marcescens* mutants affected in antibiotic and pigment production. This indicated that there was at least one other regulatory mechanism for the production of these secondary metabolites in this bacterium. This leads onto the aims of this project.

1.8 AIMS

It is clear from the above that very little is known about the regulation of pigment production in *S.marcescens*. In addition little research has focused on the production of the carbapenem antibiotic by this bacterium. Investigating the production of these secondary metabolites may not only help answer some of the questions concerning the evolutionary significance of these natural products, but might ultimately also be of clinical importance. As mentioned briefly above (discussed in more detail in section 3.0), work I carried out before starting this project pointed towards a regulatory link between prodigiosin and carbapenem production in *S.marcescens*. Using the genetic systems perfected in *Ecc*, I proposed to carry out a molecular genetic investigation of the putative pleiotropic regulator Rap. In doing so I hoped to discover new themes in the regulation of secondary metabolites.

MATERIALS AND METHODS

2.0 BACTERIAL STRAINS, PLASMIDS, COSMIDS AND BACTERIOPHAGES

The bacterial strains used in this study, are listed in table 2.1. The plasmids, cosmids and bacteriophages used in this study are listed in table 2.2.

TABLE 2.1
BACTERIAL STRAINS

Strain	Characteristics	Antibiotic resistance markers	Source	Reference
<i>Escherichia coli</i> strains				
DH1	F ⁻ , <i>recA1</i> , <i>endA1</i> , <i>th-1</i> , <i>hsdR17</i> , <i>gyrA96</i> , (<i>r_K⁻m_K⁻</i>), <i>supE44</i> , <i>relA1</i>		P. Reeves	Maniatis <i>et al.</i> , (1982)
TG1	F (<i>lac-pro</i>), <i>supE</i> , <i>thi</i> , F' <i>traD36</i> , <i>proAB</i> , <i>lacI^q</i> , Δ M15, <i>ecoK</i>		P. Reeves	Carter <i>et al.</i> , (1985)
MC1061	F- <i>ara</i> Δ 139 Δ (<i>ara-leu</i>) 7696 <i>galE15 galK16</i> Δ ac) X74 <i>rps</i> (<i>str^R</i>) <i>hsdR2</i> (<i>r_K-m_K</i> +) <i>mcrA mcrB1</i>		D.Hodgson	Wertman <i>et al.</i> , (1986)
CC118 (λ pir)	<i>araD139</i> , Δ (<i>ara, leu</i>) 7697, Δ <i>lacX</i> 74, <i>phoA20</i> , <i>galE</i> , <i>galK</i> , <i>thi</i> , <i>rpsE rpoB</i> , <i>araEam</i> , <i>recA1</i> (λ pir)		S. McGowan	Herrero <i>et al.</i> , (1990)
ESS	β -lactam super sensitive		P.Williams (provided by Smith-Kline Beecham)	Bainton <i>et al.</i> , 1992b)
<i>Erwinia carotovora</i> subsp. <i>carotovora</i> strains				
SCRI 101	Wild type		M. Holden	Scotish Crop Research Institute (SCRI)
SCRI102	Wild type		M. Holden	SCRI
SCRI103	Wild type		M. Holden	SCRI
SCRI105	Wild type		M. Holden	SCRI
SCRI106	Wild type		M. Holden	SCRI

(Table 2.1 cont) Strain	Characteristics	Antibiotic resistance marker	Source	Reference
SCRI109	Wild type		M. Holden	SCRI
SCRI112	Wild type		M. Holden	SCRI
SCRI113	Wild type		M. Holden	SCRI
SCRI114	Wild type		M. Holden	SCRI
SCRI115	Wild type		M. Holden	SCRI
SCRI116	Wild type		M. Holden	SCRI
SCRI117	Wild type		M. Holden	SCRI
SCRI118	Wild type		M. Holden	SCRI
SCRI120	Wild type		M. Holden	SCRI
SCRI121	Wild type		M. Holden	SCRI
SCRI122	Wild type		M. Holden	SCRI
SCRI123	Wild type		M. Holden	SCRI
SCRI124	Wild type		M. Holden	SCRI
SCRI125	Wild type		M. Holden	SCRI
SCRI126	Wild type		M. Holden	SCRI
SCRI127	Wild type		M. Holden	SCRI
SCRI130	Wild type		M. Holden	SCRI
SCRI132	Wild type		M. Holden	SCRI
SCRI135	Wild type		M. Holden	SCRI
SCRI139	Wild type		M. Holden	SCRI
SCRI144	Wild type		M. Holden	SCRI
SCRI149	Wild type		M. Holden	SCRI
SCRI152	Wild type		M. Holden	SCRI
SCRI155	Wild type		M. Holden	SCRI
SCRI166	Wild type		M. Holden	SCRI
SCRI169	Wild type		M. Holden	SCRI
SCRI171	Wild type		M. Holden	SCRI
SCRI172	Wild type		M. Holden	SCRI
SCRI174	Wild type		M. Holden	SCRI
SCRI192	Wild type		M. Holden	SCRI
SCRI193	Wild type		S.Jones	Forbes and Pérombelon, (1985)
SCRI198	Wild type		M. Holden	Scotish Crop Research Institute

(Table 2.1 cont) Strain	Characteristics	Antibiotic resistance marker	Source	Reference
SCC3193	Wild type		M. Holden	Swedish Culture Collection
ATTn10 (also known as GS101)	Wild-type ATCC39048 (restrictionless derivative). Carbapenem producer ¹ .		G.Salmond	Bainton <i>et al.</i> , (1992b)
ATM101	ATTn10, <i>hor::kan^R</i> marker exchange mutant	Kan ^R		This study
ATM102	ATTn10, <i>hor::kan^R</i> marker exchange mutant	Kan ^R		This study
ATM103	ATTn10, <i>hor::kan^R</i> marker exchange mutant	Kan ^R		This study
ATM104	ATTn10, <i>hor::kan^R</i> marker exchange mutant	Kan ^R		This study
ATM105	ATTn10, <i>hor::kan^R</i> marker exchange mutant	Kan ^R		This study
GB3	ATTn10. <i>carR::lacZ</i>	Kan ^R	G.Bosgelmez	Unpublished
GBH3	GB3, <i>hor::kan^R</i>	Kan ^R		This study
GB7	ATTn10. <i>carA::lacZ</i>	Kan ^R	G.Bosgelmez	Unpublished
GBH7	GB7, <i>hor::kan^R</i>	Kan ^R		This study
MR1	ATTn10. <i>carI::lacZ</i>	Kan ^R	M. Rivet	Unpublished
MRH1	MR1, <i>hor::kan^R</i>	Kan ^R		This study
MS1	Lac ⁻		M.Sebiahia	Unpublished
RJP116	HC131 Rex ⁻ (EMS ²)	Amp ^R , LamB	P. Reeves	Reeves, (1991)
<i>Erwinia carotovora</i> subspecies <i>atroseptica</i> strains (<i>Eca</i>)				
SCRI1043	Wild type		M. Holden	SCRI
SCRI39	Wild type		M. Holden	SCRI
<i>Erwinia chrysanthemi</i> strains (<i>Ech</i>)				
SCRI1066	Wild type		M. Holden	SCRI
EC16	Wild type		M. Holden	SCRI

(Table 2.1 cont) Strain	Characteristics	Antibiotic resistance marker	Source	Reference
<i>Erwinia carotovora</i> subspecies <i>betavasculorum</i> SCRI479	Wild type		M. Holden	SCRI
<i>Serratia marcescens</i> strains				
ATCC39006	Wild-type, carbapenem producer		lab stock	Bycroft <i>et al.</i> , (1987)
<i>Sbon</i>	Wild type, carbapenem non-producer		G. Stewart	Unpublished
S6	Wild type, clinical isolate	Multidrug reistant	G.P.C. Salmond	Livermore, (1992)
NT2	<i>S.marcescens rap</i> mutant (EMS ² ;pMUT13)	Kan ^R		This study
NT5	<i>S.marcescens rap</i> mutant (EMS ² ;pMUT13)	Kan ^R		This study
NT6	<i>S.marcescens rap</i> mutant (EMS ² ;pMUT13)	Kan ^R		This study
NT7	<i>S.marcescens rap</i> mutant (EMS ² ;pMUT13)	Kan ^R		This study
NT8	<i>S.marcescens rap</i> mutant (EMS ² ;pMUT13)	Kan ^R		This study
NT9	<i>S.marcescens rap</i> mutant (EMS ² ;pMUT13)	Kan ^R		This study
NTM1	<i>S.marcescens rap::kan^R</i> marker exchange mutant	Kan ^R		This study
NTM2	<i>S.marcescens rap::kan^R</i> marker exchange mutant	Kan ^R		This study
NTM3	<i>S.marcescens rap::kan^R</i> marker exchange mutant	Kan ^R		This study
NTM4	<i>S.marcescens rap::kan^R</i> marker exchange mutant	Kan ^R		This study
NTP1	<i>S.marcescens pcp_{sm}::kan^R</i>	Kan ^R		This study
NTP2	<i>S.marcescens pcp_{sm}::kan^R</i>	Kan ^R		This study
NTP3	<i>S.marcescens pcp_{sm}::kan^R</i>	Kan ^R		This study
NTP4	<i>S.marcescens pcp_{sm}::kan^R</i>	Kan ^R		This study

(Table 2.1 cont) Strain	Characteristics	Antibiotic resistance marker	Source	Reference
<i>Enterobacter agglomerans</i> ATCC59046	Wild type		K. Flint	
<i>Proteus mirabilis</i> SK1205	Wild type		K. Flint	
<i>Yersinia enterocolitica</i> WA-C	Wild type, serotype 0:8 plasmidless derivative		K. Flint	
<i>Salmonella typhimurium</i> ATCC14028s	Wild type		P.Marsh	

KEY :	1 : 1 Carbapen-2-em-3- carboxylic acid 2 : ethyl methyl sulphonate (EMS)
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TABLE 2.2

PLASMIDS, COSMIDS AND BACTERIOPHAGES

Plasmid	Characteristics	Plasmid phenotype	Source	Reference
pTroy9	pLAFRB <i>malK</i> ::IS-3, LamB	Tc ^R	P. Reeves	DeVries <i>et al.</i> , (1984)
pMUT13	pBR322:: <i>lamB</i> ⁺	Kan ^R	P. Reeves	Clement <i>et al.</i> , (1982)
pSMG4	pUC19+ <i>carR</i>	Tc ^R	S.McGowan	McGowan <i>et al.</i> , (1995)
pACYC177	Multi-copy cloning vector	Amp ^R , Kan ^R	S.McGowan	Cohen and Chang, (1978)
pNJ5000	IncP, Tra ⁺	Tc ^R	S.McGowan	Grinter, 1983
pKNG101	<i>SacB</i> Marker exchange vector	Str ^R	S.McGowan	Kaniga <i>et al.</i> , (1991)
pKNG312R	pKNG101+2368 bp <i>DraI</i> - <i>SalI rap</i> :: <i>kan</i> ^R fragment from pNRT312R	Str ^R , Kan ^R		This study
pKNG324P	pKNG101 + 3555 bp <i>EcoRV-SalI pcp_{sm}</i> :: <i>kan</i> ^R fragment from pNRT324P	Str ^R , Kan ^R		This study
pKNG25H	pKNG101 + 3714 bp <i>EcoRV-SalI hor</i> :: <i>kan</i> ^R fragment from pNTC25K	Str ^R , Kan ^R		This study
pRK2013	mobilising vector	Tra ⁺ , Kan ^R		Ditta <i>et al.</i> , 1980
pBR322	Multi-cloning vector	Amp ^R , Tc ^R	Amersham Int	Bolivar <i>et al.</i> , 1977

(Table 2.2 cont) Plasmid	Characteristics	Plasmid phenotype	Source	Reference
pBR325	Multi-cloning vector	Cm ^R , Tc ^R	D.Hodgson	Bolivar <i>et al.</i> , 1977
pNRT345	pBR325 + <i>rap</i> , <i>pcp</i> , <i>orf1</i> on 4.5kb <i>HindIII</i> - <i>Sall</i> fragment from pNRT370	Cm ^R , Tc ^R		This study
pNRT322	pBR325 + 2.2 kb <i>HindIII</i> - <i>EcoRV</i> fragment from pNRT370	Cm ^R , Tc ^R		This study
pNRT321	pBR325 + 2.1 kb <i>EcoRI</i> - <i>EcoRV</i> fragment from pNRT370	Cm ^R , Tc ^R		This study
pNRT316	pBR325 + 1.6 kb <i>Sall</i> - <i>EcoRV</i> fragment from pNRT370	Cm ^R , Tc ^R		This study
pNRT314	pBR325 + 1.4 kb <i>EcoRV</i> fragment from pNRT370	Cm ^R , Tc ^R		This study
pNRT330	pBR325 + 3.1 kb <i>Sall</i> - <i>EcoRV</i> fragment from pNRT370	Cm ^R , Tc ^R		This study
pACYC184	Multi-copy cloning vector	Tc ^R , Cm ^R	S.McGowan	Cohen and Chang, (1978)
pNRT324	pACYC184 + ' <i>orfY rap</i> , <i>pcp_{Sm}</i> , <i>orf1</i> ' on 2.3kb <i>EcoRV</i> - <i>Sall</i> fragment from pNRT345	Cm ^R		This study
pNRT324P	pNRT324, <i>pcp_{Sm}::kan^R</i>	Cm ^R		This study
pNRT312	pACYC184 + <i>rap</i> , ' <i>orfY</i> ' on 1.2 kb <i>AseI</i> - <i>Sall</i> fragment from pNRT324	Cm ^R		This study
pNRT312R	pNRT312, <i>rap::kan^R</i>	Cm ^R		This study
pNRT312B	pNRT312, <i>BspHI</i> endfill, <i>rap</i> '	Cm ^R		This study
pNRT313	pACYC184 + <i>pcp_{Sm}</i> , <i>orf1</i> ' on 1.3kb <i>AseI</i> fragment from pNRT324	Cm ^R		This study
pNTC35	pACYC184 + <i>hor_{Ec}</i> on 3.5 kb <i>EcoRV</i> fragment from pTC51	Cm ^R		This study
pNTC25	pACYC184 + <i>orf1'</i> _{<i>Ec</i>} , <i>hor_{Ec} pcp_{Ec}</i> on 2.5 kb <i>EcoRV</i> - <i>Sall</i> fragment from pNTC35	Cm ^R		This study
pNTC25K	pNTC25 <i>hor::kan^R</i>	Cm ^R , Kan ^R		This study

(Table 2.2 cont) Plasmid	Characteristics	Plasmid phenotype	Source	Reference
pNTC12	pACYC184+ 1.2kb <i>EcoRV</i> - <i>SalI</i> fragment from pNTC35	Cm ^R		This study
pSF6	multi-copy cosmid cloning vector, <i>cos</i> , <i>mob</i>	Sp ^R ,Str ^R	P. Reeves	Selvaraj <i>et al.</i> , (1984)
pNRT300	pSF6+ <i>rap</i> ¹	Sp ^R ,Str ^R		This study
pNRT370	pSF6+ 7kb <i>HindIII</i> fragment from pNRT300	Sp ^R ,Str ^R		This study
pNRT3200	pSF6+ 15-20 kb <i>BamHI</i> fragment from pNRT300	Sp ^R ,Str ^R		This study
pNRT104	pSF6+Prodigiosin biosynthetic cluster ¹	Sp ^R ,Str ^R		This study
pTC51	pSF6+ <i>hor</i> _{Ec} ²	Sp ^R ,Str ^R	T.Cox	Cox, (1995)
pTC52	pSF6+ <i>hor</i> _{Ec} ²	Sp ^R ,Str ^R	T.Cox	Cox, (1995)
Phage	Characteristics	Phenotype	Source	Reference
M13mp18	Sequencing vector		Amersham Int.	Messing and Vieira, (1982)
M13mp19	Sequencing vector		Amersham Int.	Messing and Vieira, (1982)
φKP	SCRI193 bacteriophage		I. Toth	Toth (1991); Toth <i>et al.</i> , (1993)
φKP-ATM101	φKP propagated on ATM101 <i>hor::kan</i> ^R			This study

KEY 1: Cosmid isolated from a <i>Serratia marcescens</i> strain ATCC39006 chromosomal DNA library constructed in pSF6 (N.Thomson un published). 2: Cosmids isolated from a <i>Ecc</i> ATCC39048 chromosomal DNA library constructed in pSF6 (Cox, 1995)
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2.1 GROWTH AND MAINTENANCE OF BACTERIAL STRAINS AND PHAGE

Media and solutions used for routine growth and maintenance of bacterial and phage stocks are listed in table 2.3. Media and solutions were sterilised by autoclaving at 121°C for 15 minutes. Items shown in brackets were added to the growth medium after autoclaving from sterile stocks. Media was solidified by the addition of 1.5%

(w/v) Bacto agar. Antibiotics and media supplements listed in table 2.4 were prepared in ELGA water, filter sterilised unless otherwise stated and added to the growth medium after autoclaving and cooling of the medium.

Serratia marcescens, *Erwinia carotovora* subspecies *carotovora* (*Ecc*) and *Escherichia coli* strains were maintained on NB Agar (NBA) plates, at 4°C, for up to 3 months. Stocks for long term storage were grown in liquid culture, under the appropriate conditions, and frozen at -70°C in 2x freezing solution. All phage stocks were stored at 4°C over a few drops of chloroform.

TABLE 2.3
MEDIA AND SOLUTIONS

Medium or Solution	Constituent(s) per Litre
Nutrient broth (NB)	13 g Oxoid nutrient broth
NB agar (NBA)	NB, 15 g Bacto agar
2YT	16 g Bacto tryptone 10 g Bacto yeast extract 5 g NaCl
Nutrient agar (NA)	28 g Oxoid nutrient agar
Blood agar	5% (w/v) Sheeps blood 1.5% (w/v) Bacto agar
Phage Buffer	10 mM Tris base 10 mM MgSO ₄ 0.01% (w/v) gelatine (pH 7.4)
SOC	20 g Bacto tryptone 5 g Bacto yeast extract (10 ml 1M NaCl) (2.5 ml 1M KCl) (10 ml 1M MgSO ₄) (10 ml 1M MgCl ₂) (20 ml 1M glucose) (pH 6.8)

(Table 2.3 cont)	
Medium or Solution	Constituent(s) per Litre
50x Phosphate Buffer	350g K ₂ HPO ₄ 100g KH ₂ PO ₄ (pH6.9-7.1)
Minimal medium (MM)	(20ml 50x phosphate buffer) (10ml 10% (w/v) (NH ₄) ₂ SO ₄) (10ml 1% (w/v) MgSO ₄ .7H ₂ O) (10ml 20% glucose or sucrose as stated)
High sucrose Minimal medium (for Marker Exchange)	(20ml 50x phosphate) (10ml 10%(w/v) (NH ₄) ₂ SO ₄) (10ml 1% (w/v) MgSO ₄ .7H ₂ O) (200ml 50% (w/v) sucrose-prewarmed)
Protease Indicator Medium (PRT)	13 g Oxoid nutrient broth 30 g Oxoid gelatine
Protease indicator plate developer	4M (NH ₄) ₂ SO ₄
Pectate Lyase Indicator Medium (PEL)	(5 ml 20 % (w/v) Bacto yeast extract) (10 ml 10 % (w/v) (NH ₄) ₂ SO ₄) (1 ml 1M MgSO ₄ .7H ₂ O) (10 ml 50 % (v/v) glycerol) (250 ml 2 % (w/v) PGA, pH 5.5) (200 ml Pel phosphate buffer)
Pectinase indicator plate developer	7.5 % (w/v) copper acetate
Cellulase Indicator Medium (Cel)	10g carboxymethylcellulose (Sigma) (25 ml 20 % (w/v) Bacto yeast extract) (4 ml 50% (v/v) glycerol) (20 ml 50x phosphate buffer) (10 ml 10 % (NH ₄) ₂ SO ₄) (10 ml 1% (w/v) MgSO ₄ .7H ₂ O)
Cel indicator plate developer	0.2 % (w/v) Congo Red (Sigma) 1M NaCl 1M HCl

(Table 2.3 cont)	
Medium or Solution	Constituent(s) per Litre
Z-buffer	8.52 g Na ₂ HPO ₄ (anhydrous) 6.24 g NaH ₂ PO ₄ .2H ₂ O 0.75 g KCL 0.25 g MgSO ₄ .7H ₂ O 2.7 ml mercaptoethanol (pH 7.0)
Freezing Medium (2x)	126 g K ₂ HPO ₄ (anhydrous) 0.9 g sodium citrate 0.18 g MgSO ₄ .7H ₂ O 3.6 g KH ₂ PO ₄ (anhydrous) 1.8 g (NH ₄) ₂ SO ₄ 88 ml glycerol
Triton lysis mix	0.1 % (v/v) Triton X-100, 50 mM Tris-HCl 50 mM EDTA (pH 8.5)
TES	50 mM Tris-HCl, 5 mM EDTA, 50mM NaCl (pH 8.0)
STE	25% (w/v) sucrose, 5 mM EDTA, 50 mM Tris-HCl, (pH 8.0)
TBE (10x)	108g Tris base, 55g Boric acid, 9.3g EDTA
TE	10 mM Tris-HCl, 1 mM EDTA, (pH 8.0)
TE buffer	50 mM Tris-HCl, 50 mM EDTA, (pH 8.0)
PEG precipitation solution	10% PEG 6000 (w\v) 12.5M NaCl

(Table 2.3 cont) Medium or Solution	Constituent(s) per Litre
Acrylamide stock (40%)	38% (w/v) acrylamide 2% (w/v) <i>NN</i> -methylenebisacrylamide filtered through 2% (w/v) Amberlite mixed resin, stored at 4°C in the dark.
Denaturation solution	87g NaCl 20g NaOH
Neutralisation solution	87g NaCl 121g Tris-HCl (pH8.0)
SSC (20x)	175g NaCl 88g Na CH ₃ COOH (Sodium citrate)
Denherts solution (50x)	10g Ficol 10g Polyvinylpyrrolidone 10g Bovine serum albumen (Sigma; stored -20°C)
SSPE (20x)	174g NaCl 31.2g NaH ₂ PO ₄ · H ₂ O 7.4g EDTA (Adjust to pH 7.4 with ~21 ml NaOH, 10M)
Prehybridisation solution (radio active probe)	(require only 10 ml per filter) 250 ml 20x SSPE 100 ml 50x Denherts 100 ml 10 % SDS
Wash 1 (6x SSC)	10 ml SDS (10 %) 300 ml SSC (20x) (use only 150 ml/wash)
Wash 2 (2x SSC)	10 ml SDS (10 %) 100 ml SSC (20x) (use only 150 ml/wash)
Wash 3 (0.5x SSC)	10 ml SDS (10 %) 10 ml SSC (20x) (use only 150 ml/wash)

(Table 2.3 cont)	
Medium or Solution	Constituent(s) per Litre
Prehybridisation solution (DIG probe)	(require 20 ml/filter) 250 ml 5xSSC 0.1 % (w/v) N-Lauroylsarcosine, Na salt (Sigma) 1 % (w/v) Blocking reagent from kit stored -20°C
DIG Buffer 1- (Malate Buffer)	8.8 g NaCl 11.6 g Malic acid (pH 7.5)
DIG Buffer 2	10% Block (Boehringer Mannheim kit component) in Malate buffer (DIG Buffer 1)
DIG Buffer 3	(100 ml of 1M NaCl) (100ml of 1M Tris pH 9.5)

TABLE 2.4
SUPPLEMENTS TO GROWTH MEDIA

Supplement	Stock Solution (mg/ml) or (w/v)	Final Concentration (µg/ml)	Abbreviation
Sodium ampicillin	5	50	Amp
Kanamycin sulphate	5	50	kan
Streptomycin sulphate	10	100	Str
Tetracycline ¹	1	10	Tc
Spectinomycin sulphate	5	50	Sp
Chloroamphenicol ¹	2.5	25	Cm
5-bromo-4-chloro-3-indolyl -D-galacto-pyranoside ²	2% (w/v)	As directed	X-Gal
Isopropyl-1-thio-β- D-galactosidase	2.5% (w/v)	As directed	IPTG
N-(3-oxo-hexanoyl)-L- homoserine lactone ³	1	1	OHHL (a.k.a HSL)

KEY 1: Tc and Cm were made up in 50 % (v/v) ethanol and stored at -20°C.
 2: X-Gal was made up fresh in dimethyl formamide (DMF), and was not filter sterilised.
 3: OHHL was not filter sterilised. Storage was in 10 µl aliquots at -20°C.

2.2 THE PREPARATION, ANALYSIS AND TREATMENT OF DNA

2.2.1 PREPARATION OF DNA

2.2.1.1 SMALL SCALE PREPARATION OF PLASMID DNA (MINI-PREP)

This method is based on the alkaline lysis method as described by Maniatis *et al.*, (1982) with the following adaptation. To ensure a high yield of DNA with all cosmid constructs based on the low copy vector pSF6, the initial culture volume used was increased to 4ml.

2.2.1.2 LARGE SCALE PREPARATION OF PLASMID DNA (MAXI-PREPS) FROM *Escherichia coli* (RUN ON A CAESIUM CHLORIDE GRADIENT)

This method was used to prepare high quality DNA for cloning and sequencing. The method is based on Maniatis *et al.*, (1982). A 250 ml culture (2YT) was grown in a 2 litre flask, with shaking and with the appropriate antibiotic selection, overnight (New Brunswick Incubator Shaker, 37°C, 275 rpm). Cells were recovered by centrifugation (MSE Hi-Spin 21, 8,000 rpm, 20 min, 4°C). The supernatant was discarded and the cell pellet resuspended in 20 ml TES and transferred to an oakridge tube. Cells were recovered by centrifugation (MSE Hi-Spin 21, 10,000 rpm, 10 min, 4°C). The supernatant was discarded and the cell pellet resuspended in 10 ml STE. The cell suspension was then frozen (30 min, -70°C or at -20°C until required).

After thawing on ice, 1 ml of the lysozyme solution (10mg/ml in 250 mM Tris-HCl [pH 8.0]) was added and the contents of the tube mixed by inversion. The tube was place on ice for 10 min, with occasional mixing by inversion. 2.5 ml 0.5M EDTA was added, and the contents of the tube mixed. The tube was placed on ice for 10 min, with occasional mixing and then 16 ml of triton lysis mix was added quickly using a pipette, and the tube shaken vigorously. The tube was left on ice until lysis was observed (approximately 20 min). The sample was centrifuged (MSE Hi-Spin 21, 19,000 rpm, 30 min, 4°C) and the supernatant decanted, carefully so as not to disturb the pellet, into a 50 ml measuring cylinder through a double layer of muslin. 28.5g of CsCl was added

and the contents of the measuring cylinder mixed by rocking. The sample was placed at 65°C to facilitate dissolving of CsCl. 2 ml of ethidium bromide (5mg/ml) was added and the volume of the sample brought to 40 ml by the addition of TES. The sample was transferred to an oakridge tube and placed on ice for 30 mins.

The sample was centrifuged (MSE Hi-Spin 21, 19,000 rpm, 15 min, 4°C), and the supernatant was decanted into a 45 ml opaque Quick Seal tube (Beckman), using a 20 ml syringe attached to 0.8 mm bore needle, containing glass wool to trap any debris. Tubes were balanced by the addition of CsCl solution (71.28 % w/v) and filled to the top with paraffin oil. Tubes were sealed (Beckman tube sealer) and centrifuged (Beckman L8-70M Ultracentrifuge, Vti50 rotor, 45,000 rpm, 22°C, 16 h).

Plasmid DNA was visualised using a long wave transilluminator (330 nm) to avoid damaging the DNA. The DNA was removed by inserting one 0.8 mm bore syringe needle into the top of the tube to prevent a vacuum forming and another just above the plasmid band taking great care not to take up any chromosomal DNA whilst plasmid DNA was removed. The solution containing plasmid DNA was transferred to an opaque 5 ml Quick Seal tube (Beckman). Tubes were balanced and sealed as described previously. Samples were centrifuged (Beckman L8-70M Ultracentrifuge, Vti65 rotor, 65,000 rpm, 22°C, 5 h) and the plasmid DNA was removed as described previously.

Ethidium bromide was removed from the solution containing plasmid DNA by extraction with NaCl-saturated isopropanol. An equal volume of NaCl-saturated isopropanol was added, the sample mixed and centrifuged (Micro Centaur, high speed, 30 s), and the organic phase removed. The extraction was repeated 5 times. The sample was transferred to an oakridge tube and DNA was precipitated by the addition of 2 volumes sterile water and 6 volumes of ethanol. The sample was mixed by vortexing and incubated at -20°C overnight. DNA was recovered by centrifugation (MSE Hi-Spin 21, 19,000 rpm, 15 min, 4°C). The DNA pellet was washed in 70% (v/v) ethanol, dried at 55°C, resuspended in 500 µl TE (Table 2.2a), and stored at -20°C.

2.2.1.3 LARGE SCALE PREPARATION OF CHROMOSOMAL DNA

The method adapted from Maniatis *et al*, (1982) by P.J.Reeves (pers. comm.). A 25 ml culture was grown with shaking in 2YT broth for 16h (New Brunswick Incubator Shaker, 30°C, 275 rpm). The culture was transferred to an oakridge tube and centrifuged (MSE Hi-Spin 21, 10,000 rpm, 15 min, 4°C). The cell pellet was resuspended in 25 ml 1M NaCl and centrifuged (MSE Hi-Spin 21, 10,000 rpm, 15 min, 4°C). The supernatant was discarded and the cell pellet frozen (-70°C, 30 min). The cell pellet was thawed on ice and resuspended in 16 ml TE buffer 2 ml of freshly prepared lysis solution (250mM Tris-HCl, 2mg/ml lysozyme [sigma] pH 8.0) was added and the contents of the tube mixed by inversion. The tube was left on ice for 5-10 min until cell lysis was observed. Proteinase K (Sigma, final concentration of 20 µg/ml) and 500 µl of 10% SDS (w/v) was added, and the contents of the tube mixed by inversion. The tube was incubated at 65°C, until the cell lysate had cleared (usually 20 min). 3 ml of 5M sodium perchlorate was added mixed by inversion. The tube was incubated at 65°C for a further 15 min. The volume of the cell lysate was brought to 40 ml by the addition of resuspension solution and the tube was mixed by inversion. The cell lysate was extracted twice with phenol, and twice with chloroform : isoamyl alcohol (24:1), using a trimmed, blue, Gilson tip to remove the DNA and avoid shearing it..

The upper aqueous layer was poured into an ethanol rinsed glass beaker and 2 volumes of ethanol added by pouring down the side of the beaker. DNA was spooled with an ethanol rinsed glass rod and left to air dry. DNA was dissolved in 2-5ml of TE and 5 µl chloroform added. DNA was stored at 4°C.

2.2.1.4 SMALL SCALE PREPARATION OF CHROMOSOMAL DNA

For routine chromosomal DNA preparation this proved to be a very useful protocol for all bacterial strains. A 10ml culture in 2YT was grown overnight and then the cells harvested by centrifugation (Labor 50-M, 4,500 rpm, 10 min) and resuspended in 10ml of TE. Freshly prepared lysozyme solution (2ml, 5mg/ml in 0.25M Tris-HCl

pH 8.0) was added and the sample placed on ice for 5min for the cells to lyse.

Proteinase K (200 μ l, 10 mg/ml, Sigma) was added along with SDS (300 μ l, 10% stock w/v, Sigma) and the sample was incubated for 1hr at 65°C before the addition of 3ml of 5M sodium perchlorate and a further 1hr incubation at 65°C. The sample was allowed to cool on ice and phenol extracted twice and then once with chloroform. 500 μ l of this was placed in a 1.5ml Eppendorf tube and 1ml of ethanol added and mixed by inversion. The chromosomal DNA was recovered by centrifugation and dried in a vacuum. The DNA was dissolved in 150 μ l of TE, which generally provided enough DNA for 4-5 restriction digests. DNA samples were stored at 4°C.

2.2.2 SPECTROPHOTOMETRIC MEASUREMENT OF DNA

Purified DNA was routinely assayed in quartz cuvettes on a Philips PU 8720 spectrophotometer following the method of Maniatis *et al.*, (1982).

2.2.3 PHENOL/CHLOROFORM EXTRACTION AND ETHANOL PRECIPITATION OF DNA

This method is based on that described by Maniatis *et al.*, (1982). The solution containing the DNA was combined with an equal volume of phenol (buffered with Tris-HCl (pH 8.0) fisons) and vortexed to form an emulsion. The sample was centrifuged (MSE Micro Centaur, high speed, 13,000 rpm, 1 min) and the upper aqueous layer removed without disturbing the interface (small volume DNA samples, such as 20 μ l enzyme digests, were diluted by the addition of 80 μ l of TE to ease the removal of the upper aqueous phase). An equal volume of chloroform was added and vortexed to form an emulsion. The upper aqueous layer was removed following centrifugation. The DNA was recovered by the addition of 0.1 volume 3 M NaOAc (pH 4.8) and two volumes ethanol, mixed by vortexing and chilled at -20°C for 30 min. DNA was recovered by centrifugation (MSE Micro Centaur, high speed, 13,000 rpm, 15 min) and the supernatant discarded. The DNA pellet was washed in 70% ethanol (v/v) and again recovered by

centrifugation. The ethanol was removed and aspirated to remove any residue. The DNA was dried (55°C, 5min) and resuspended in TE.

2.2.4 RESTRICTION ENDONUCLEASE TREATMENT OF DNA

Restriction endonuclease digests were usually performed in a final volume of 20 µl. The digestion of chromosomal and plasmid DNA was performed according to manufacturers recommendations (BRL, New England Biolabs or Amersham) most usually for an hour at 37°C. "Double enzyme" digests were performed in the same tube if the restriction buffers were similar. In this case a two step reaction using the lower salt concentration buffer first, and then altering the salt concentration by the addition of 1M NaCl etc (heat treatment was used to denature the first enzyme). The sample was then re-incubated with the second enzyme for another hour. If the buffers were incompatible the DNA was cut with one enzyme for an hour then extracted with phenol chloroform, recovered by ethanol precipitation and resuspended in 15µl of TE before the second restriction enzyme digest. All restriction digests were analysed by agarose gel electrophoresis and if a band was required for cloning it was purified by Geneclean (section 2.3).

2.2.5 PHOSPHATASE TREATMENT OF DOUBLE STRANDED DNA (LINEAR)

Re-circularisation of vector DNA following restriction endonuclease digestion was prevented by de-phosphorylation with calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim) according to suppliers instructions, with the following modifications. Following digestion of the vector DNA, CIP treatment was carried out in a total volume of 30µl by the addition of sterile ELGA water. The DNA was purified by agarose gel electrophoresis and recovered by Geneclean (section 2.3).

2.2.6 "END REPAIR" OF LINEAR DOUBLE STRANDED DNA

Restriction endonuclease digested DNA or sonicated DNA that generated 3' or 5' overhanging ends, if necessary, were removed by "End repair", using T4 DNA Pol 1

(BRL) or DNA Pol 1 "Klenow" fragment (BRL) as directed by manufacturers instructions. dNTP mix (dATP, dCTP, dGTP and dTTP) was obtained from Boehringer Mannheim and used at a final concentration of 5mM diluted in TE.

Sonicated or ragged ends were treated as above with the exception of using both DNA Pol 1 "Klenow" fragment and T4 DNA Pol 1. All end repaired samples were incubated at room temperature for 30 min and the DNA was purified by phenol extraction, recovered by ethanol precipitation and resuspended in the appropriate volume of TE.

2.2.7 DNA LIGATION

The method was essentially that described by Maniatis *et al.*, (1982). Vector DNA was digested with the restriction endonuclease(s) of choice, treated with CIP (calf intestinal alkaline phosphatase; Boehringer Mannhiem), and resuspended in an appropriate volume of TE buffer. The DNA to be ligated was digested with the compatible restriction enzyme(s), run on and excised from an agarose gel, Gene cleaned and resuspended in the minimal volume of TE (~ 10-15µl). Vector and insert DNA were mixed in a 1:3 ratio and appropriate amounts of DNA ligation buffer (BRL, 5x), ATP (final concentration 1mM, filter sterilised) and T4 DNA ligase (1-2U ligase per ligation, BRL) was added, and the volume brought to 20µl with sterile ELGA water. Samples were incubated overnight at 16°C.

2.2.8 DNA STANDARDS FOR AGAROSE GEL ELECTROPHORESIS

Size Markers for agarose gel electrophoresis of all DNA analysed in this study(kb):
1kb Ladder (BRL)

12.216	6.108	1.018	0.220
11.198	5.090	0.517	0.201
10.180	4.072	0.506	0.154
9.162	3.054	0.396	0.134
8.144	2.036	0.344	0.075
7.126	1.636	0.298	

2.2.9 AGAROSE GEL ELECTROPHORESIS

Agarose (0.7% final concentration) was melted in TBE (table 2.3) containing ethidium bromide added (final concentration 0.1µg/ml). The molten gel was poured into a Bio-Rad sub or mini cell with a gel comb. When set this was submerged in 1xTBE containing ethidium bromide (final concentration 0.1µg/ml). DNA samples were mixed with 0.2 volumes loading buffer (50% glycerol, 0.25% bromophenol blue) and run at between 12-100 volts for 1-16hr as appropriate. Size of DNA fragments was determined by comparison with 1kb ladder commercial size markers (BRL). DNA was visualised on a longwave UV transilluminator (330nm) and photographed using Polaroid 665 film on a short wave UV transilluminator (260nm). DNA to be Gene cleaned was not exposed to short wave UV to prevent DNA damage.

2.3 ISOLATION OF DNA FROM AGAROSE GELS USING GENE CLEANTM (STRATECH)

DNA fragments generated by restriction digest or sonication were separated on an agarose gel. The DNA of interest was visualised on a longwave transilluminator and cut from the gel using a scalpel blade. The DNA was recovered from the gel according to manufacturers instructions (Geneclean II kit BIO-101).

2.4 TRANSFORMATION OF CELL BY FOREIGN DNA

2.4.1 CALCIUM CHLORIDE TRANSFORMATION OF *Escherichia coli*

A 50ml culture of *E.coli* cells to be made competent were grown to an OD₆₀₀ of 0.5 (550nm Philips PU 8720 spectrophotometer) in 2YT broth (New Brunswick Incubator Shaker, 37°C, 275 rpm). The culture was divided into two universal tubes and centrifuged (Labor 50-M, 4,500 rpm, 10 min, 4°C) and the cell pellet was resuspended in 25 ml 0.1M MgCl₂ (chilled on ice). The cell suspension was centrifuged as before and the pellet was resuspended in 25ml (ice cold) 0.1M CaCl₂. The cells were then pelleted again and resuspended in 3ml (ice cold) 0.1M CaCl₂. Cell competence

was improved by chilling the cell suspension on ice for at least 2 hrs (Maniatis *et al.*, 1982).

DNA (typically 10-50 ng) was added to 200 μ l of competent cells, and mixed by inversion. The mixture was chilled on ice for 40 min, followed by "heat shock" at 42°C for 2 min. When gene expression time was required, 1ml of 2YT was added and the cell suspension incubated for 1hr prior to spreading on selective media. Expression time was increased to 2-3 hrs for *Serratia* cells. Competent untransformed cells were used as the control.

2.4.1 ELECTROPORATION

Electroporation was found to be the most efficient way to transform *Erwinia carotovora* subspecies *carotovora* and *Serratia marcescens*. Electroporation was also used to transform *Escherichia coli* when high efficiency was required for example when introducing ligated non-supercoiled DNA. All bacterial strains to be transformed were prepared in the same way as detailed below.

A 10ml culture was grown to mid-log phase (*E.coli* : New Brunswick G24 Incubator Shaker, 37°C, 275 rpm; *Ecc* strains and *S.marcescens*; 30°C, 275 rpm) in 2YT. The culture was centrifuged (Labor-50M, 4,500 rpm, 10 min) and the cell pellet was washed three times with 10ml of sterile ice cold ELGA water. The cell pellet was finally resuspended in 200 μ l sterile ELGA water and placed on ice prior to use. DNA (5-50 ng) was added to a 60 μ l aliquot of cells, mixed by pipetting, and the mixture transferred to an electroporation cuvette (0.2 cm electrode, Bio-Rad). The mixture was shaken down to form an even layer over the cuvette base. The cuvette was placed on ice prior to electroporation.

Electroporation was carried out using a Bio-Rad Gene Pulser according to manufacturers instructions. The pulse delivered was 25 μ F, 2.5kV and 200 Ω . Immediately after pulsing 1 ml 2YT medium was added to each sample. If gene expression time was required the cell suspension was transferred to a fresh tube and

incubated at the appropriate temperature (*E.coli*: 37⁰C; *Ecc* and *S.marcescens*: 30⁰C) for 30 min-3hr, depending on the bacterium, prior to spreading on selective media.

2.5 CONJUGAL TRANSFER ('PATCH MATING')

Routinely colonies from the donor (usually *E.coli* strains DH1 or CC118 containing pRK2013 or pKNG101 marker exchange derivatives, respectively) and recipient strains were mixed together on a 2YT agar plate, using a sterile wire loop, and incubated overnight at 30⁰C. A sterile wire loop was used to remove cells from the 'patch mating' which were then streaked out onto minimal media (to select against the auxotrophic *E.coli* donor) containing sucrose and the relevant antibiotics to select for transfer of the plasmid but against any unconjugated recipient. A further streak onto the minimal media plates was found to be necessary to remove the persistent *E.coli* donor cells

For tri-parental mating it was found that increased efficiency was attained by growing the relevant strains up in universals (10ml nutrient broth containing the appropriate antibiotics, 30⁰C for *Ecc* and *S.marcescens*, 37⁰C *E.coli*, overnight) and spotting 10µl of each bacterial strain on top of each other on a 2YT agar plate. Transconjugants were selected as above.

2.6. THE POLYMERASE CHAIN REACTION (PCR)

Polymerase chain reaction (PCR) was used to amplify a gene(s) of interest from chromosomal DNA samples, for cycle sequencing, and to make DNA probes for Southern blots. The method was essentially that described for the "basic" PCR reaction of Erlich, (1989).

A series of dilutions of chromosomal and plasmid samples were prepared in sterile water (Fisons, ultrapure). Oligonucleotide primers for PCR were designed as 17-25 bp oligomers, some of which included a restriction site to aid cloning (see appendix for all the primers designed in this study). The primer DNA was resuspended in sterile water (Fisons) such that the concentration of primer was ~1.5 ng per ml. Reaction components

were added to a siliconised Eppendorf tube (500 µl, Sigma) taking care to use a fresh Eppendorf tip for each addition. Reactions were carried out in a total volume of 50 µl.

Standard PCR reaction mix:-

10 µl each primer
3 µl 100 mM MgCl₂ (Promega)
1 µl diluted chromosomal or plasmid DNA^a
4 µl dNTP mix (dATP, dCTP, dGTP, dTTP; Boehringer Mannheim ,1.25 mM)
5 µl Taq polymerase buffer (Promega)
17 µl sterile water (Fisons, ultrapure)

^a(Chromosomal DNA was diluted 1:10 if made by a large scale DNA prep, plasmid DNA made by maxi prep was generally diluted 1:200)

The reaction mix was overlaid with 50 µl paraffin oil to prevent evaporation. Control reactions were also prepared (final volume 50µl made up with sterile water) which either lacked template DNA or primers . The PCR machine used was a Omnigene, Hybaid and the standard program used is detailed below:

PCR cycling parameters;**Stage 1**

94°C for 5 mins - denaturation

Hold at 85 °C - to allow for the addition of 0.3µl Taq DNA polymerase (Promega)

*? °C for 2 min - Primer annealing temperature

72 °C for 2 min - for extension along the template by Taq

(1 cycle)

Stage 2

94 °C for 30s - denaturation

*? °C for 30s - annealing

72 °C for 40-60s - to extend, time depending on predicted product size

(35 cycles)

Stage 3

*? °C for 1 min - final annealing

72 °C for 2min - final extension

(1 cycle)

*? Annealing temperatures for primers were calculated according to the Wallace rule (Ikatura *et al.*, 1984) and are quoted in the appropriate section where the relevant primer is mentioned. Where two primers with different annealing temperatures were used the lower annealing temperature was used.

All products were run on an agarose gel and Gene cleaned before use. Note that products PCR labelled with DIG (section 2.7.2) ran higher on an agarose gel than an identical unlabelled product.

2.7 SOUTHERN HYBRIDISATION

2.7.1 TRANSFER OF DNA FROM AN AGAROSE GEL TO A NYLON MEMBRANE

The DNA to be probed was digested with restriction endonucleases and run on an agarose gel. After electrophoresis the agarose gel was placed in denaturing solution (150ml in a polypropylene sandwich box) for 2x15 minutes. In order to neutralise the DNA the procedure was repeated in Neutralising solution (150ml 2x15 minutes).

The gel was then placed on filter paper (Whatman 3MM) on a glass sheet. The filter paper dipped into a tray containing 200ml of 10xSSC. Nylon membrane (Hybond N, Amersham) was cut to the size of the gel and placed on top. Three layers of filter paper (Whatman 3MM) cut to size and soaked in 10xSSC and at least two inches of paper tissue was placed on top of the filter, making sure there were no air bubbles between the gel, the nylon and the filter paper. A heavy weight such as a house brick was placed on top. This was left for at least 4hrs (usually overnight), the DNA was fixed to the nylon membrane by exposure to shortwave UV (UV transilluminator 260nm) for 3min then the membrane was dried and stored between two sheets of Whatman 3MM paper at room temperature.

2.7.2 SYNTHESIS OF DNA PROBES FOR SOUTHERN BLOTS

DNA to be used as a probe, generated by restriction endonuclease digestion or more frequently by PCR, was always purified from an agarose gel using 'Gene clean' before use (section 2.3). DNA probes were either labelled with [α^{32} P]dCTP, using the

random primed DNA labelling kit (Boehringer Mannheim) or using the non-radioactive digoxigenin-11-dUTP (DIG) kit (Boehringer Mannheim) according to manufacturers instructions. Non-radioactive probes were generally made using PCR because of the more efficient labelling of the probe (see section 2.6). Note that products PCR labelled with DIG run higher on an agarose gel than an identical unlabelled product.

2.7.3 HYBRIDISATION OF RADIOACTIVE PROBES TO FILTERS

Hybridisation of Southern filters with a DNA probe was carried out essentially according to Maniatis *et al.*, (1982). Pre-hybridisation was carried out for a minimum of 1 hr in pre-hybridisation solution (table 2.3), hybridisation solution was the same as the pre-hybridisation solution except with the addition of the DNA probe. All the hybridisation and wash steps were carried out using in a hybridisation oven (Hybaid Mini Oven, MKII), according to manufacturers instructions. Hybridisation of the [$\alpha^{32}\text{P}$] dCTP labelled DNA probe with the Southern filter was carried out for 12-16h. The temperature of pre-hybridisation and the hybridisation step was 55°C unless otherwise stated.

Three post-hybridisation washes were used; 10 minutes for each of the wash solutions 1, 2 and 3 (wash solutions are detailed in table 2.3). After post-hybridisation washes wet filters were blotted dry with 3MM Whatman paper, wrapped in Cling film, and exposed to X-ray film (Fuji medical) at -70°C overnight, or longer (up to a week) for weak signals.

2.7.4 HYBRIDISATION OF DIG PROBES TO SOUTHERN FILTERS

Hybridisation of Southern blot filter with a digoxigenin-11-dUTP labelled (DIG) DNA probe was carried out essentially according to manufacturers instructions. Detection solutions were modified to minimise the "background" signal. DIG Buffer 2 contained an increased amount of blocking reagent (1.5%; table 2.3).

Pre-hybridisation and hybridisation were carried out in a hybridisation oven (Hybaid Mini Oven, MKII), according to manufacturers instructions. The pre-hybridisation solution is detailed in table 2.3. The hybridisation solution consisted of

pre-hybridisation solution plus the DIG labelled probe. Both hybridisation steps were carried out at a temperature of 55°C unless otherwise stated, and once the DNA probe was added to the filter it was usually left overnight. After the overnight incubation with the probe the filter was immersed in Wash 1 (table 2.3) at 55°C for 2x15 min. Detection steps were carried out at room temperature in a sandwich box. The filter was washed in DIG buffer1 for 1 min and then immersed in 100 ml of DIG buffer 2, with gentle shaking for 30 mins. The 'blocked' filter was immersed in 40 ml DIG buffer 2 containing 4µl of anti-DIG antibody for 30 min. The filter once washed in DIG buffer 1 (2x100ml for 15 min each) was equilibrated in DIG buffer 3 for 2 min and then placed on an acetate sheet (Lloyd Paton). 0.5 ml of DIG buffer 3 containing 1:100 dilution of Lumigen PPD detection reagent (kit component) was distributed all over the filter. The filter was then sandwiched between another acetate sheet and exposed to X-ray film (Fuji medical) at room temperature. Exposure time varied between 1-16h.

2.8 SEQUENCING DNA

2.8.1 PREPARATION OF DNA FOR RANDOM SEQUENCING

The DNA fragment to be sequenced was obtained by restriction endonuclease(s) digestion and isolated from an agarose gel by Gene clean. The DNA fragment was self-ligated to generate closed circles and /or chains (depending on the fragment ends).

The ligated DNA (in a volume of 30 µl) was sheared using a cup horn sonicator (Heat Systems Ultrasonics W-380) filled with water to a depth of approximately 3 cm, with the sample clamped 1 mm above the probe. The DNA was sonicated for two bursts (maximum output, 80s). If necessary between bursts the sample was briefly centrifuged (MSE Micro Centaur, high speed, 3s), replaced on ice, and the water surrounding the probe replaced to aid cooling.

Ragged ends generated by sonication of DNA were "End repaired" (section 2.2.6). Repaired sonicated DNA was analysed by gel electrophoresis (1.5% agarose gel) and DNA fragments (in the size range 300-1,000 bp) were isolated by Gene clean

2.8.2 LIGATION OF END REPAIRED DNA INTO M13

M13 (50ng, mp18 or mp19, New England Biolabs) DNA was digested with restriction enzyme *Sma*I at 30°C for 2hr, then treated with CIP (section 2.2.5) and purified from an agarose gel. End filled sonicated DNA fragments (section 2.8.1) were ligated into the cut M13 vector and *E.coli* strain TG1 was transformed with the ligated DNA by the calcium chloride transformation procedure. No expression time is required and heat shocked cells were mixed with 3mls of molten 2YT top agar and poured onto freshly prepared 2YT agar plates containing IPTG 25µl and X-Gal 25µl, (blue/white selection, white indicating a disruption to the β-galactosidase gene likely to be a ligated insert) once set this was incubated overnight at 37°C.

2.8.3 LIGATING WHOLE FRAGMENTS INTO M13

To sequence whole DNA fragments: DNA to be cloned was excised using the appropriate restriction enzyme and purified using Gene clean. The fragment was ligated into M13 cut with restriction enzymes which generated compatible ends and treated with CIP (section 2.2.5). *E.coli* strain TG1 was transformed, by the calcium chloride procedure, with the products of the ligation and templates were prepared as for random sequencing (section 2.8.4).

2.8.4 ISOLATION OF PLAQUES, PREPARATION OF M13 LYSATES AND TEMPLATE DNA

White plaques were cut from the top agar using a short tipped pasteur pipette. The plaque was then 'blown out' into an Eppendorff containing 50µl of phage buffer (table 2.3). M13 lysates prepared in this way were stored at 4°C for up to two weeks.

An overnight culture of *E.coli* strain TG1 was diluted 1\100 in 2YT broth and 1.5ml aliquots were dispensed into sterile phage tubes. 25µl of the M13 lysate was added to infect the TG1 and incubated at 37°C (New Brunswick G24 Environmental Incubator Shaker). After 5hrs incubation the culture was centrifuged and the supernatant

transferred to another tube containing 200µl PEG precipitation solution (table 2.3), this was inverted 5x and left overnight at 4°C for the phage to precipitate.

Recombinant phage DNA was recovered by centrifugation (MSE Microcentaur , 13,000 rpm, 20min) and the supernatant removed by aspiration. A second spin was carried out (MSE Microcentaur , 13,000 rpm, 5min) to remove any residual PEG solution before 100µl of TE was added. The M13 template DNA was then phenol extracted and recovered by ethanol precipitation (section 2.2.3) to be resuspended in 25µl of TE and stored at -20°C.

2.8.5 SEQUENCING RANDOM M13 CLONES USING SEQUENASE

All sequencing reactions were performed according to manufacturers instructions (Sequenase V2.0 US,Biochemical) using 7µl of the M13 template DNA (section 2.8.4). A maximum of 10 templates were sequenced at any one time. Sequence reactions were stored at -20°C until required and were heated at 80°C for 12 min prior to being loaded onto an acrylamide sequencing gel.

2.8.6 CASTING AND RUNNING A POLYACRLYAMIDE SEQUENCING GEL

The Biorad, BR102 sequencing 'rig' was prepared and assembled according to manufacturers instructions using 0.4 mm spacers. The acrylamide gel mixes used are as follows:-

1x TBE gel mix (white)	75 ml 40% acrylamide stock(table 2.3) 50 ml 10x TBE (table 2.3) 230g urea (BRL) Filtered and stored in the dark at 4°C Made up to 500ml with water
5x TBE gel mix (blue)	30 ml 40% acrylamide stock 100 ml10x TBE 92 g Urea (BRL) 10 mg bromophenol blue Filtered and stored in the dark at 4°C Made up to 200ml with water

To cast a gel a plug of acrylamide gel was made between the two gel plates. The plug consisted of 8ml of 5x TBE mix to which 24 μ l of TEMED (Sigma) and 24 μ l of ammonium persulphate (20% freshly prepared, Biorad) was added. This was taken up in a 10ml pipette and run out slowly along the bottom of the glass plates. The gel mix was taken up by capillary action and dried to leave a firm plug in \sim 20min. To form the main body of a gradient gel, 75ml of the 1xTBE (white) and 12ml of the 5xTBE (blue) acrylamide gel mixes were poured into separate washed beakers. TEMED (150 μ l) and ammonium persulphate (150 μ l) were added to the white gel mix and 24 μ l of TEMED and ammonium persulphate were added to the blue gel mix. These were briefly mixed by swirling and the 60ml of the white mix was taken up in a 100ml syringe and set aside. 10ml of the white gel mix was taken up in a 25ml pipette followed by 12ml of blue gel mix, two bubbles were drawn into the pipette to mix the two solutions. The acrylamide mix in the 25ml pipette was run down the centre of the assembled gel rig (held at an angle of \sim 45 $^{\circ}$) followed quickly by all the white gel mix in the syringe. A well forming comb was inserted and the gel allowed to set (typically 1hr).

The gel plates were inserted into the lower reservoir tray and these reservoirs filled with 1xTBE (table 2.3). The gel comb was removed and the wells flushed with 1xTBE to remove any unpolymerised acrylamide. Sequencing reactions were (section 2.8.5) heated at 80 $^{\circ}$ C for 12min and loaded onto the gel (2 μ l using Hamilton syringe). The reactions were electrophoresed for half an hour after the first dye front had run off the bottom of the gel (constant power 100W). The gel was fixed (10% v/v acetic acid 15min), transferred to Whatman filter paper covered in Clingfilm and dried (Bio-Rad Slab Gel Drier, 80 $^{\circ}$ C for 30min). The Clingfilm was removed and the gel exposed to X-ray film (Fuji Medical, overnight).

DNA sequence was analysed with the aid of a digitizer (Science Accessories Corporation) and Microgenie computer package (Beckman). The sequence was then analysed on various computer packages (see section 2.9).

2.8.7 EXTENSION REACTIONS AND SEQUENCING THROUGH COMPRESSIONS

If it was necessary to read more than the usual 50-250bp of nucleotide sequence downstream of the primer binding site, then any residual template reaction mix was re-run on another acrylamide gel, with the gel running time increased to twice the standard time (typically 8hr). In order to keep the temperature of the gel at the optimum the 1xTBE (table 2.3) buffer was changed half way through the run. This method usually made it possible to read a further 50-100 bp of sequence. Alternatively reaction conditions were altered as specified by manufacturers instructions (Sequenase Version 2.0). Compressions were dealt with as specified by manufacturers instructions using dITP nucleotide mixes.

2.8.8 CYCLE SEQUENCING

2.8.8.1 PRIMER DESIGN

Primers were designed as for manufacturers instructions (7100 Delta Taq Cycle sequencing kit; USB).

2.8.8.2 CYCLE SEQUENCING REACTIONS AND PCR PROTOCOL

The cycle sequencing reactions were carried out exactly as specified by the manufactures instructions (7100 Delta Taq Cycle sequencing kit; USB). The primer DNA was resuspended in sterile water (Fisons) such that the concentration of primer was ~1.5ng per ml. Reaction components were added to a siliconised Eppendorf tube (500µl, Sigma) taking care to use a fresh Eppendorf tip for each addition. All reactions were carried out in a Omnigene, Hybaid PCR machine. The reaction mix was as follows:-

Reaction mix:	5 µl Cycle sequencing Primer 2 µl Taq reaction buffer (kit component) 5 µl PCR product (PCR amplified gene of interest, purified by Gene clean and resuspended into 20 µl sterile water) 2 µl of each of two of the following; dTTP, dGTP or dCTP - (depending on primer design) 2 µl Taq (1:7 dilution in Taq dilution buffer; kit components) 0.5 µl ³⁵ S dATP
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Cycle sequencing PCR protocol**First round of the protocol (primer labelling step):**

94°C for 2 min (denaturation temperature)

?^a °C for 15s (annealing temperature)

72 °C for 15s (extension temperature)

(1 cycle)

94 °C for 30s

?^a °C for 15s

72 °C for 15s

(45 cycles)

Hold at room temperature

? ^a Primer annealing temperatures varied depending on the primer (see appendix).

The reactions were then taken out of the PCR machine and 3.5 µl of it was dispensed into each of 4 wells on a micro titre plate. The four wells had already been filled with 4 µl of one of the four dideoxynucleotides; ddATP, ddTTP, ddCTP or ddGTP. A hole had also been cut in the micro titre plate to allow the temperature probe of the PCR machine to go through the plate. 50 µl of paraffin was added to each well and the plate centrifuged for 10s at 1000 rpm. The plate was then replaced in the PCR machine for the second round of the protocol. The primers were designed such that after extension they would have been extended by usually 8-10 nucleotides (including a minimum of four ³⁵S dATP nucleotides), elevating the primer annealing temperature such that annealing and extension could both occur at the same temperature (72°C), as detailed below.

Second round of the PCR protocol:

94 °C for 2 min

72 °C for 2.5 min (annealing and extension temperature)

(1 cycle)

94 °C for 30s
72 °C for 2 min
(35 cycles)

Stop buffer was then added to the reactions. These could then be run directly on a polyacrylamide gel (section 2.8.6) or could be stored at -20 °C until required.

2.9 COMPUTER ANALYSIS OF NUCLEOTIDE SEQUENCE AND PROTEIN SEQUENCE

2.9.1 MICROGENIE

All sequence compilation and primary analysis was performed on this Beckman package (Queen and Korn, 1984). Further analysis was then performed on the following computer packages.

2.9.2 UWGCG (UNIVERSITY OF WISCONSIN GENETICS COMPUTER GROUP) PROGRAMS USED:

REFORMAT Converts entered sequence into the correct format for use with GCG programs.

FRAMES: For the identification of possible open reading frames (Orfs).

TRANSLATE: Translates DNA sequence into protein sequence.

PUBLISH: Presents DNA and protein sequence in a publishable format.

PILEUP: Multiple sequence alignment program (DNA or protein).

PRETTY: Uses the pileup output file, and calculates a consensus sequence from the multiple lineup.

SEQED: Sequence could be entered directly into the computer, and edited, using this program.

MAPPLOT: This program constructs a restriction map for the entered DNA. For subcloning this was an invaluable tool.

2.9.3 WORLD WIDE WEB PROGRAMS USED, WITH INTERNET ADDRESS:

PEDRO'S: http://www.fmi.ch/biology/rt_1.html

Catalogue of many of the available biomolecular tools on the Inter Net.

ALLALL: http://cbrg.inf.ethz.ch/subsection3_1_1.html

For the construction of dendrograms

BLAST SEARCH:

http://ulrec3.unil.ch/software/EPFLBLAST_form.html

http://www.ncbi.nlm.nih.gov/Recipon/bs_seq.html

http://dot.imgen.bcm.tmc.edu:9331/cgi-bin/seq-search/blast_form_local.pl

Various databases for DNA and protein homology searches and the retrieval of published sequences.

GENBANK: http://ncbi.nlm.nih.gov/genbank/query_form.html

Database for DNA and protein homology searches and the retrieval of published sequences.

OWL: <http://www.biochem.ucl.ac.uk/bsm/dbbrowser/OWL/OWL.html>

Database for the retrieval of published sequences.

pI/Mw: http://expasy.hcuge.ch/ch2d/pi_tool.html

Isoelectric point and molecular weight determination.

PSORT: <http://psort.nibb.ac.jp/form.html>

Predicts protein cellular localisation and the presence of possible bacterial signal sequences (Von Heijne, 1986; Nakai and Kanehisa, 1991).

TMPRED: http://ulrec3.unil.ch/software/TMPRED_form.html

Protein membrane topology predictions (Hoffman and Stoffel, 1993; Nakai and Kanehisa, 1991).

All DNA sequenced during the course of this study was analysed comprehensively using the above programs. All of these programs have detailed instructions in their use and can be accessed easily using an Inter Net server such as the

Mosaic server used at Warwick. GCG does have a database search function (FASTA), however the programs found on the Inter Net were found to be more up to date and have a more comprehensive information base.

2.10 MARKER EXCHANGE

The gene to be exchanged was inactivated as described in the appropriate section. The inactivated, recombinant gene, was then ligated into pKNG101 (appendix; Kaniga *et al.*, 1991) and *E.coli* strain CC118 (λ *pir*) transformed by the calcium chloride method. Transformants were selected on NB containing the appropriate antibiotics, usually kanamycin and streptomycin. A tri-parental patch mating was then performed (section 2.5) with the recipient cells, a helper strain of *E.coli* (carrying plasmid pNJ5000) and the *E.coli* strain CC118 (λ *pir*) containing the recombinant marker exchange plasmid. Colonies were streaked out on minimal selective media. This was repeated twice to ensure that there was no carry over of the parental strains. Bacteria recovered after the patch mating were grown overnight in 10ml of broth. 10 μ l of a 1:100 dilution of this culture was then spread on high sucrose minimal media (table 2.3) supplemented with the appropriate antibiotic to select for the mutant allele. The chromosomal DNA was prepared from a number of surviving colonies and was analysed by Southern blot, with an appropriate DNA probe, in order to confirm the genotype.

2.11 PREPARATION AND USE OF HIGH TITRE ϕ KP LYSATES

The preparation and the use of high titre ϕ KP lysates was carried out as described in Toth, (1991) and Toth *et al.*, (1993). Lysates were titred by spotting serial dilutions (10^{-2} , 10^{-4} , 10^{-6} and 10^{-8}) on top of agar lawns seeded with 200 ml of *Ecc* strain SCRI193.

2.12 POTATO TUBER VIRULENCE ASSAY

This method was used to test the ability of the *Ecc hor* marker exchange mutant to cause soft rot and was performed as described by Walker *et al.*, (1994b). Inoculated tubers were incubated in a sealed plastic box at 25°C for 24, 48, 72 and 96 hrs. The

extent of rotting was determined by scooping out the soft rotted potato flesh and weighing it.

2.13 CARBAPENEM DETECTION ASSAY

Production of carbapenem by *Erwinia* and *Serratia* was detected using the *E. coli* strain ESS bioassay, this strain of *E.coli* is super sensitive to β -lactam antibiotics. 200 μ l of an overnight culture of the *E.coli* strain ESS was added to 4 ml of molten top agar (0.7%) and poured onto a NA plate to form a top lawn. Once set, the strain to be tested was stabbed into, with a sterile tooth pick, or spotted (5 μ l) onto this top lawn (spotting culture onto the lawn was found more sensitive). The plates were incubated overnight at 25°C. A clear zone of antibiosis or halo around the test strain indicated antibiotic production.

2.14 INDICATOR PLATE ASSAYS FOR EXOENZYME ACTIVITY

Indicator plate assays were used as a routine screen for exoenzyme activity. Bacterial strains to be tested were stabbed, with sterile toothpicks, or 5 μ l of an overnight culture of the strain was spotted onto the indicator plates and allowed to dry in. Plates were incubated at 30°C overnight. Protease indicator plates were developed as described by Hankin and Anagnostakis, (1975). Pectinase indicator plates were developed as described by Andro *et al.*, (1984). Cellulase indicator plates were developed as described by Gilkes *et al.*, (1984). The constituents for each of the three detection plates are detailed in table 2.3 along with the assay plate developing solutions.

Exoenzyme activity was observed as a clear halo was seen against a white background for protease. For pectate lyase, double rings (dark blue) surrounding a light blue halo were seen against a light blue background. For cellulase, a pale blue/red halo was seen against a dark blue background.

2.15 PLOTTING THE GROWTH CURVE OF A CULTURE

The optical density (OD) of a culture was measured at 600 nm on a Philips PU 8720 spectrophotometer, at regular intervals throughout the growth cycle, by either using a side arm flask or removing 0.5-1 ml of the culture into a cuvette. A graph of OD against time could then be plotted.

2.16 CULTURE FRACTIONATION

For the enzyme assays performed in this study, 5 ml samples of the bacterial culture were centrifuged (Labor-50M, 4,500 rpm, 4°C, 15 min) and the supernatant carefully removed and stored at -20°C in 1 ml aliquots until required.

The cell pellet was washed twice in 5 ml of the growth medium. Cells were recovered by centrifugation. After the last wash, the cell pellet was resuspended in 5 ml growth medium and transferred to a 25 ml glass beaker on ice. The sample was sonicated (MSE sonicator, 2 cm probe, amplitude 6 microns) for 3 x 30s bursts, with a cooling interval of 30s between bursts. Cell debris was removed by centrifugation (Labor 50-M, 4,500 rpm, 4°C, 15 min), and stored in 1 ml aliquots at -20°C until required. For the β -galactosidase assays performed in this study, samples of 500 μ l were taken throughout the growth curve, the complete sample was sonicated (supernatant and cellular fraction together) using a MSE sonicator, 3 mm probe, amplitude 12 microns for 3 x 30s bursts, with a cooling interval of 30s between bursts. The samples were stored at -20°C until required.

2.17 SPECTROPHOTOMETRIC ENZYME ASSAYS

2.17.1 PROTEASE (Prt) ASSAY

Protease activity was assayed according to the method of Braun and Schmitz, (1980). Samples, previously fractionated (section 2.16), were assayed using growth medium as a blank in the reaction mix. Samples were analysed using a Philips PU 8720

spectrophotometer at a wavelength of 436 nm. Activity was expressed as $\Delta A_{436}/h/ml/OD_{600}$ unit.

Protease (Prt) liquid enzyme assay - Reaction Mix	1 ml 2 % (w/v) azocasein (Sigma) prepared fresh 200 μ l 1M Tris-HCl (pH 8.0) 800 μ l sample (133 μ l of supernatant or 266 μ l of sonicate made up with H ₂ O)
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2.17.2 CELLULASE (Cel) ASSAY

Cellulase activity was assayed according to the method of Nelson, (1944) and Somogyi, (1952). Copper reagent and Colour reagent were made as described by (Collmer *et al.*, 1982). Growth medium was used as a blank. Samples (750 μ l of culture supernatant or cell sonicate + 750 μ l reaction mix) were analysed using a Philips PU 8720 spectrophotometer at a wavelength of 623 nm. Activity was expressed as $\Delta A_{623}/h/ml/OD_{600}$ unit

Cellulase (Cel) liquid enzyme assay - reaction mix	1 % solution of carboxymethylcellulose (Sigma, high viscosity) in 25 mM phosphate buffer (pH 7.0)
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2.17.3 PECTATE LYASE (Pel) ASSAY

Pectate lyase activity was assayed according to Starr *et al.*, (1977). Samples, previously fractionated (section 2.16) were assayed (22.5 μ l of culture supernatant or cell sonicate + 876 μ l reaction mix), using growth medium as a blank in the reaction mix. Samples were analysed using a Philips PU 8720 spectrophotometer at a wavelength of 235 nm. Activity was expressed as $\Delta A_{235}/min/ml/OD_{600}$ unit.

Reaction Mix	3.2 ml 0.575 % (w/v) PGA 1.13 ml ELGA water 3.45 ml Reaction buffer
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Reaction buffer	78 µl 1M CaCl ₂ 23 µl 1M Tris-HCl (pH8.5) 77 µl ELGA water
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2.17.4 β-GALACTOSIDASE (LacZ) ASSAY

The method, based on that of Miller *et al.*, (1972), was used to assess the effect of *hor::kan^R* genetic lesion on the transcription of *carR*, *carI* and *carA* genes. 25ml bacterial cultures were grown in 250ml flasks at 30°C, shaking at 300 rpm. Samples (0.5ml) were with drawn at regular intervals and fractionated to measure total β-galactosidase activity. 5-50 µl of the sample was added to Z-buffer to give a final volume of 500 µl. It was found to be extremely important to mix the constituents of the reaction mix thoroughly. The amount of sample added depended on the activity of that sample. To measure the β-galactosidase activity accurately in some cases it was necessary to reduce the amount of sample so that the colour change was not immediate. This was especially important when a large number of samples were being measured concurrently. A time of >30 min to observe a colour change was optimal. All samples were measured in triplicate. The blank consisted of 500 µl of Z-buffer.

Aliquots of ONPG (100 µl; 4 mg/ml in fresh Z-buffer) were dispensed into samples, and the time noted. Samples were incubated (37°C) until a faint yellow colour developed at which point the reactions were stopped by the addition of 250 µl of 1M Na₂CO₃, and the time noted. Samples were transferred to cuvettes and analysed on a Philips PU 8720 spectrophotometer at a wavelength of 420 nm. The β-galactosidase activity was expressed in Miller units (ΔA₄₂₀/min/ml) correcting for the blank and sample volume.

2.18 DETERMINATION OF PROTEIN CONCENTRATION

Bio-rad protein reagents were used according to manufacturers instructions. Samples were analysed using a Philips PU 8720 spectrophotometer.

2.19 PREPARATION OF CELLS FOR VIEWING UNDER A SCANNING ELECTRON MICROSCOPE (SEM)

A 10 ml overnight culture grown in NB was centrifuged (Labor 50-M, 4,500 rpm, 10 min) and resuspended in 18 ml water containing 2 ml of gluteraldehyde (25 % stock). The cells were left overnight at 4°C and then washed 5x in ELGA water. One drop of cells, diluted to varying concentrations with water, was placed on a cover slip (glued to a SEM stud with 'Dag' paint) and allowed to dry. Once dry, the cells were gold coated for 120 seconds and observed under the SEM.

***ANALYSIS OF ANTIBIOTIC AND
PIGMENT PRODUCTION BY
Serratia marcescens***

3.0 PREFACE

Prior to starting my PhD I worked for Prof. G.P.C.Salmond as a technician, working on antibiotic and pigment production by *S.marcescens*. The experiments I conducted during this period must be included here to adequately introduce the work described in this thesis. However these experiments will be restricted to the preface and will only be discussed briefly.

Various plasmids, encoding the *Escherichia coli* LamB protein, were transferred into *S.marcescens* by conjugation and electroporation. This protein acts as a receptor for coliphage λ , making derivatives of *S.marcescens* sensitive to infection by λ . This is a powerful genetic 'tool' in itself, but also facilitates transposon mutagenesis using phage carrying various transposons commonly used for the study of *Erwinia carotovora* subspecies *carotovora* (*Ecc*) in this laboratory (Salmond *et al.*, 1986; Ellard *et al.*, 1989). Various other molecular techniques of transformation and mutagenesis which had been optimised for use with *Ecc* were found to be directly transferable to *S.marcescens*.

Chemical mutagenesis using ethyl methyl sulphonate (EMS) was used to generate carbapenem mutants (*Car*⁻). One method used to assess the efficacy of the mutagenesis regime was to note the number of pigment mutants (*Pig*⁻); approximately 2-3% of the survivors of EMS mutagenesis were *Pig*⁻. To isolate *Car*⁻ mutants ~3000 colonies surviving EMS mutagenesis were picked onto the *E.coli* strain ESS bioassay plates, including a significant number of strains which were defective for pigment production. Subsequent analysis of the *Pig*⁻ mutants revealed that of the 2-3 % generated by the EMS mutagenesis a significant number of them, 10-20%, were also *Car*⁻, that is they were pleiotropic mutants. Rare spontaneous mutation events led to the concomitant reversion of antibiotic and pigment production in these mutants, implying that this phenotype was the result of a single mutation. It was thought, even with only a limited knowledge of the biochemistry of the respective pathways, that this phenotype was

unlikely to be the result of a defect in the production of a common intermediate and so it was proposed that there was a regulatory link between the two pathways. The pleiotropic phenotype was denoted Rap for the regulation of antibiotic and pigment.

Various Rap mutants (NT2, 5, 6-9) were isolated, before and during this study, by chemical mutagenesis as described above. Assuming there was a regulatory link, then these mutants would be of special interest, not only because of the coordinate control of the production of two secondary metabolites but also because carbapenem is produced constitutively throughout the growth cycle in *S.marcescens* (Bycroft *et al.*, 1988) whereas prodigiosin is a classical secondary metabolite produced late in the growth cycle (Williams and Quadri, 1980).

To investigate the Rap phenotype further a *S.marcescens* chromosomal library was constructed in the low copy number cosmid pSF6 (Selveraij *et al.*, 1984) following the method of Reeves, (1991). This library was packaged into λ and used to transduce various Rap mutants harbouring the *lamB* encoding plasmid pTroy9 (De Vries *et al.*, 1984). Cosmid pNRT300 was identified by its ability to complement the Rap mutants back to Car⁺ and Pig⁺ (figure 3.1). The library had been constructed such that every cosmid contained approximately 30-35 kb of chromosomal DNA insert. In order to investigate the regulation of antibiotic and pigment it was proposed that cosmid pNRT300 be subcloned and eventually sequenced. The possible link between antibiotic and pigment production went on to form the main impetus for this study as discussed in the next chapter.

In addition to pNRT300, I isolated other complementing cosmids using *Ecc* as a system with which to isolate the *S.marcescens* carbapenem biosynthetic genes. *Ecc* was used as a surrogate system because no *S.marcescens* single Car⁻ mutants had been isolated. This was later found to be due to the mild antimicrobial activity of prodigiosin leading to false positives on the *E.coli* strain ESS bioassay plates (Cox, 1995). *Ecc* produces an identical carbapenem antibiotic to that of *S.marcescens* (Parker *et al.*, 1982) and so it was possible that their biosynthetic systems were similar. Various classes of

Figure 3.1. The complementation of the *S.marcescens rap* mutants by cosmid pNRT300.

The photograph shows three strains of *S.marcescens*: the wild type strain (ATCC39006; Top), NT2 (a *rap* mutant; Middle) and NT2 transformed with pNRT300 (the *rap* cosmid; Bottom). The *E.coli* strain ESS bioassay (right hand side plate) shows the halo caused by the production of the carbapenem antibiotic. The lack of pigmentation for the *rap* mutant NT2 can be seen in the plate on the left hand side. Cosmid pNRT300 restores both antibiotic and pigment production to mutant NT2

Figure 3.2. In *Ecc* prodigiosin production is regulated by OHHL.

The plate has been covered with an overlay of top agar seeded with *Ecc* strain RJP116 (*carI*) which has been transformed with pNRT104. *N*-(3-oxohexanoyl)-L-homoserine lactone autoinducer (OHHL also known as HSL) or water was spotted onto the filters placed on top of the overlay. As the OHHL has diffused away from the filter the production of the red pigment prodigiosin has been induced.

FIGURE 3.1

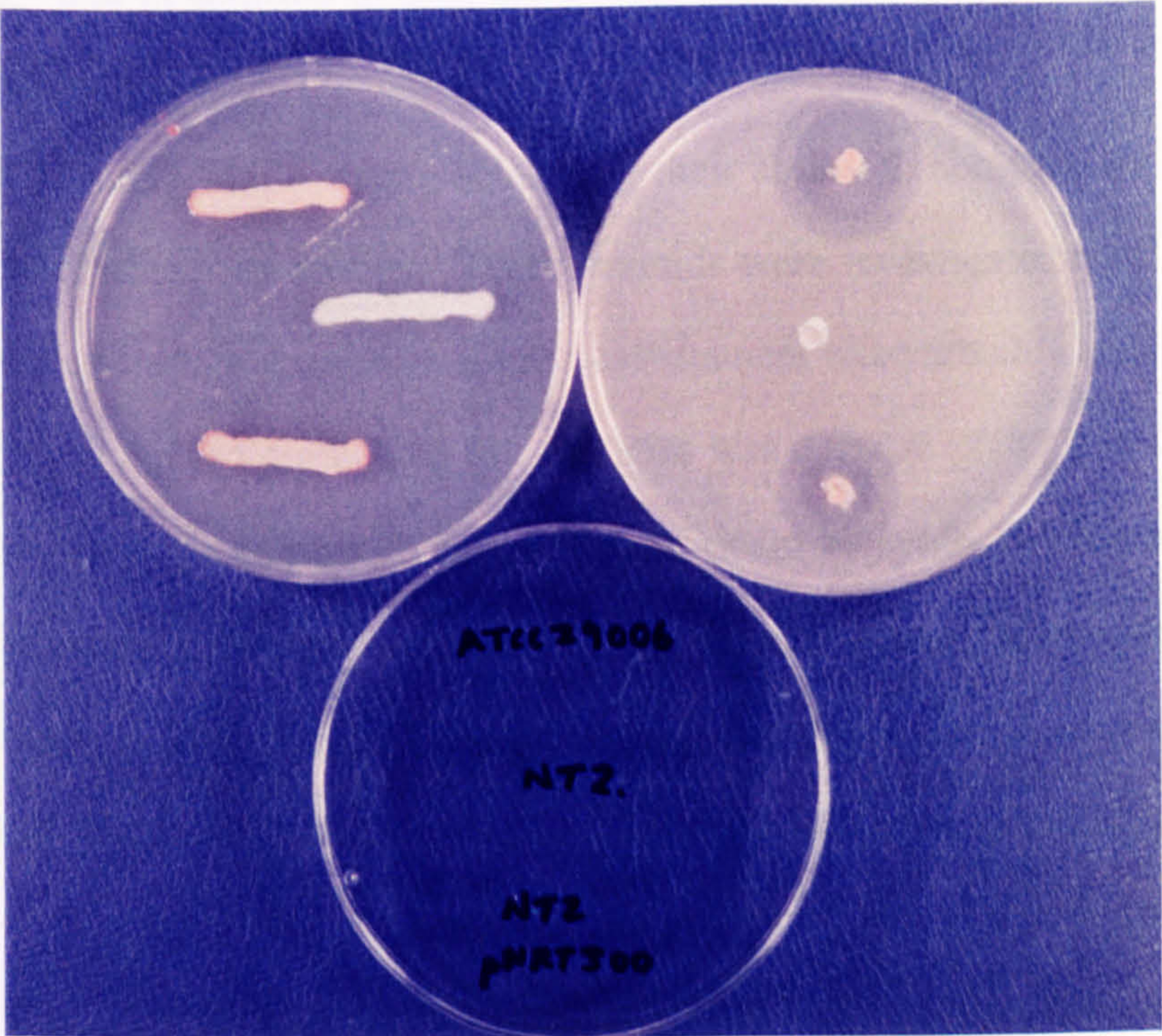
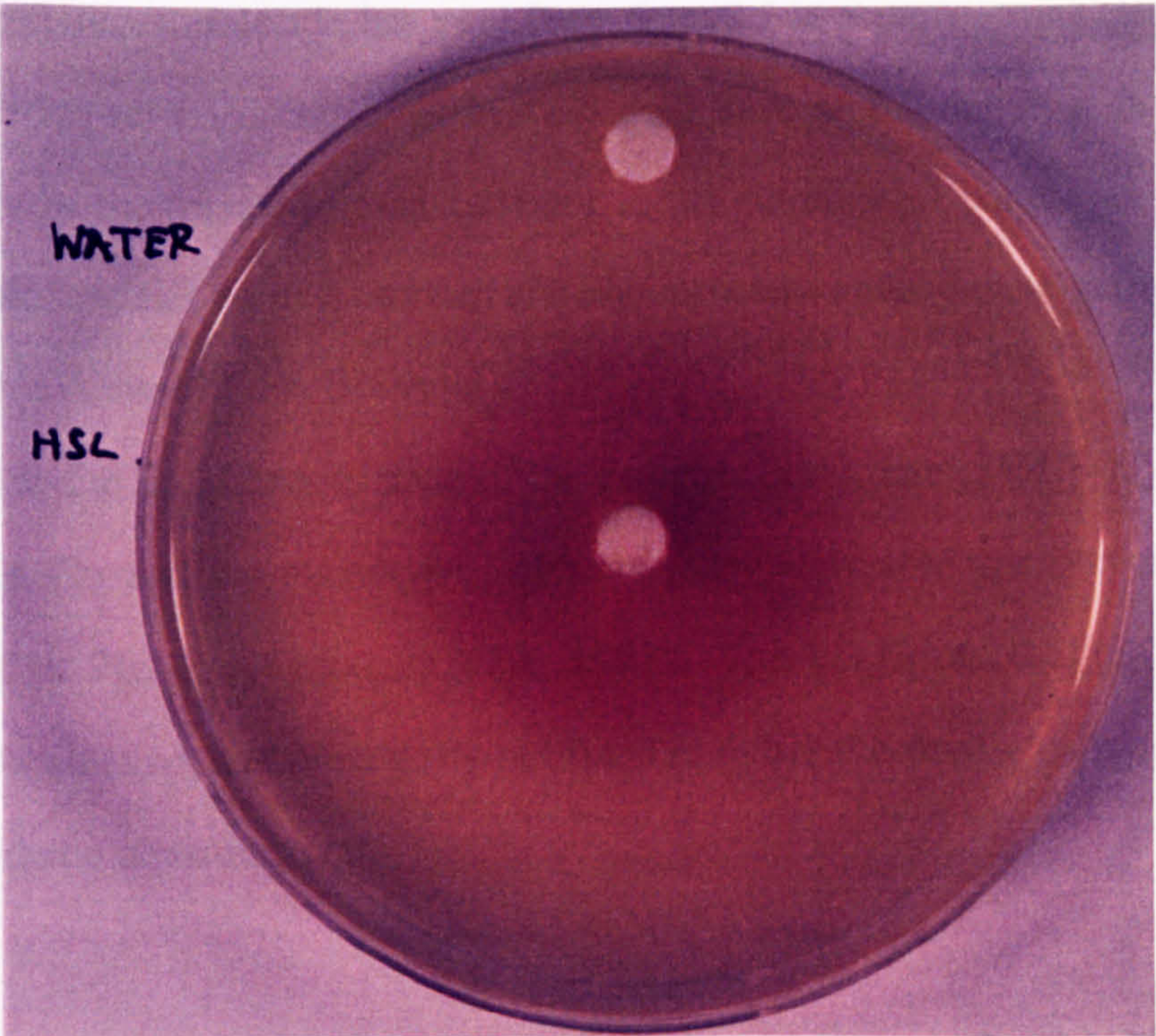


FIGURE 3.2



Ecc carbapenem mutant were available and so the *S.marcescens* chromosomal library was transduced into these mutants using phage λ . Some of the transductants isolated were found to be Car⁺ and in addition to this, there were several red colonies observed on the antibiotic selection plates.

Cosmids pNRT1 and pNRT20 were isolated by their ability to complement carbapenem production in *Ecc* Car⁻ strains. These cosmids were investigated by Cox, (1995) and were found to contain the *S.marcescens* carbapenem biosynthetic cluster. The cosmid isolated from the red pigmented *Ecc* colonies was denoted pNRT104 and was able to complement a large number of *Serratia* prodigiosin biosynthetic mutants (Cox, 1995) and so is assumed to contain all the *pig* biosynthetic genes. Many previous attempts have been made to clone the prodigiosin biosynthetic genes from *S.marcescens* with limited success as discussed in section 1.6.6. Therefore this represented the first time the *S.marcescens pig* genes had been successfully expressed in an heterologous host. Interestingly Cox, (1995) reported that the prodigiosin biosynthetic genes when introduced into *Ecc* were subject to regulation by N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), the small molecule autoinducer known to regulate the production of exoenzymes and carbapenem in *Ecc* (figure 3.2; Jones *et al.*, 1993; McGowan *et al.*, 1995). No such autoinducer molecule has ever been detected in *S.marcescens*, although the work of Cox, (1995) revealed that *S.marcescens* does have a homologue of *luxR*, which is proposed to be the transcriptional activator in the autoinducer paradigm. The prodigiosin biosynthetic genes from *S.marcescens* are currently under investigation by Tony Cox and Sophia Abassi in this laboratory.

With regard to the Rap mutants none of the cosmids other than pNRT300 could complement both the Car and Pig phenotypes, although they were able to complement their respective Car⁻ or Pig⁻ single mutants (Cox, 1995). This confirmed that the putative *rap* gene was distinct and apparently unlinked to either the prodigiosin or the carbapenem biosynthetic gene clusters.

3.1 CONFIRMATION THAT THE PIGMENT PRODUCED BY *Erwinia carotovora* SUBSPECIES *carotovora* (*Ecc*) WAS PRODIGIOSIN

The cosmid pNRT104 contained all the information required to reconstitute prodigiosin biosynthesis in *Ecc*. Before pursuing this, it was important to determine if this pigment was in fact prodigiosin. Prodigiosin displays a characteristic colour change from red/pink in an acid solution to orange when in an alkaline solution (Williams and Quadri, 1980). Either 1 drop of concentrated HCL or NaOH (10M) was added to an overnight culture of *Ecc* expressing prodigiosin. The colour change observed from this was consistent with this pigment being prodigiosin. A more definitive test was also carried out. Pigment was extracted using isopropanol saturated with NaCl (NaCl saturated isopropanol is immiscible with water). If a culture is mixed with saturated isopropanol two phases appear and the pigment, if left for several hours, is extracted and partitions to the solvent layer, which can then be removed and measured on a spectrophotometer. Prodigiosin has a characteristic absorption maximum in isopropanol of 466 nm under alkali conditions and 540 nm under acid conditions (MERCK, 1983). Extracted pigment was acidified or made basic as described above and then measured on a scanning Philips PU 8720 spectrophotometer. The two profiles measured under acid and basic conditions have been overlaid in figure 3.3. The characteristic peaks observed confirmed that the pigment produced by *Ecc*, transformed with pNRT104, was in fact prodigiosin.

3.1.1 ANALYSIS OF PIGMENT PRODUCTION BY *Erwinia carotovora* SUBSPECIES *carotovora* AND VARIOUS OTHER GENERA

To determine if the heterologous expression of prodigiosin was unique to *Ecc* and to try to gain an understanding of how the pigment might be regulated in *S.marcescens* (by analogy), pNRT104 was transferred to various *Ecc* strains by conjugation in a biparental patch mating with *E.coli* strain DH1 containing the mobilising plasmid pRK2013 (Ditta *et al.*, 1980) and cosmid pNRT104.

FIGURE 3.3

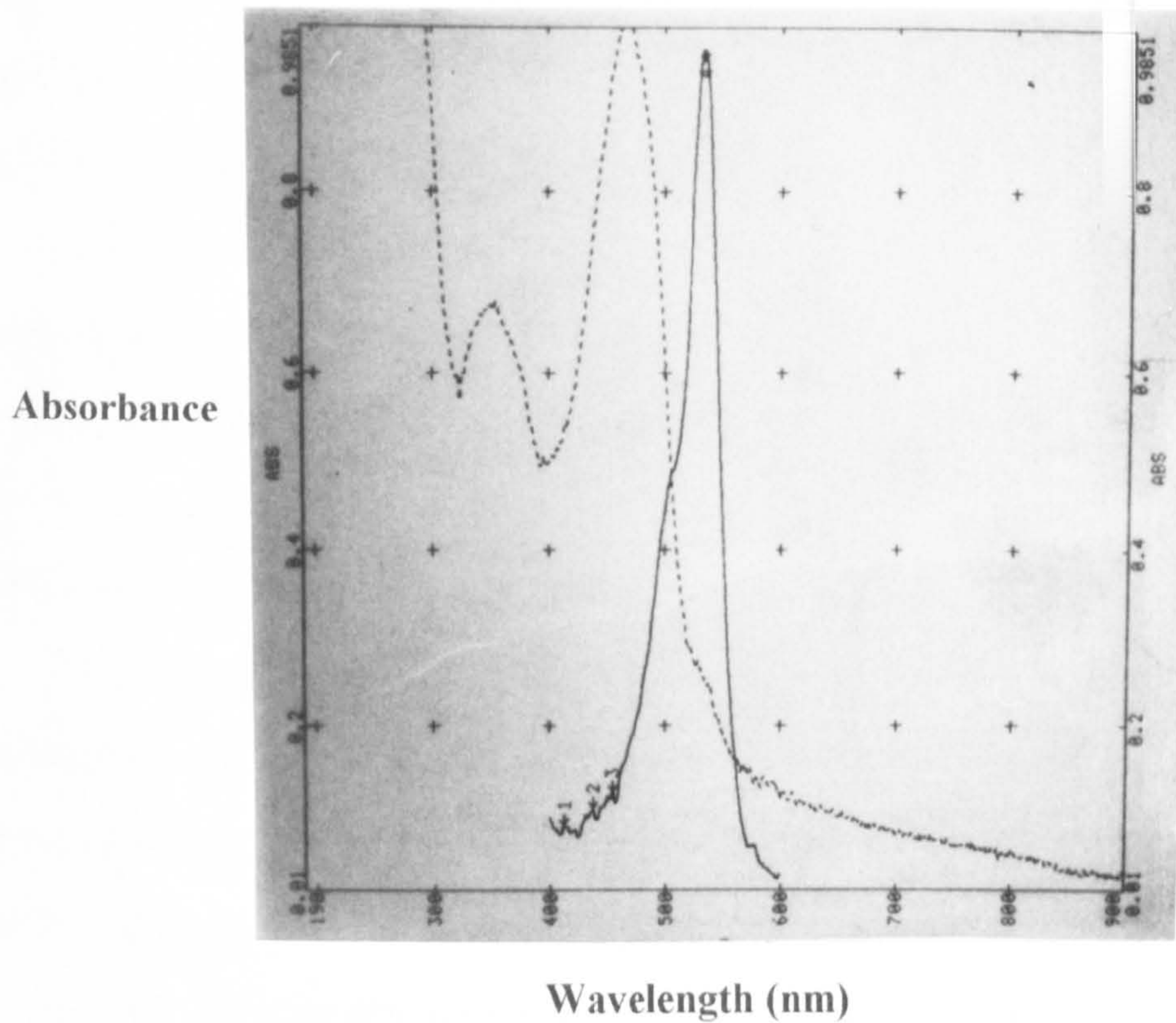


Figure 3.3 Isopropanol extraction of the red pigment expressed in *Ecc* strains carrying pNRT104. The solvent layer was removed and made acidic or basic by the addition of 1 drop concentrated HCl or a drop of NaOH (10M) respectively. The absorbance was then read on a Philips PU 8720 spectrophotometer. The two peaks of interest are at 466 nm (alkali) and 540 nm (acid) and are characteristic of prodigiosin. The readings taken under basic conditions are represented by the dotted line, whereas the solid line represents the absorbance of an acidified solution.

Transconjugants were selected on minimal media supplemented with spectinomycin and with sucrose (0.2 % final concentration) as a carbon source to select against the donor *E.coli* strain. Other bacteria in the lab culture collection were also transformed with pNRT104 by electroporation. The pigment phenotype of all the *Ecc* strains tested is recorded in table 3.1. None of the other bacterial strains (including *Escherichia coli*, *Proteus mirabilis*, *Enterobacter agglomerans* and *Pseudomonas* species) produced any observable pigment when transformed with pNRT104.

Exogenous OHHL was added to all the bacteria, apart from the *Ecc* strains tested (all of which have been found to produce autoinducer; Holden, 1996). This was to determine if OHHL was essential for the expression of pigment in these bacteria, as it was in *Ecc* strain ATCC39048 (Cox, 1995). The results were as before, no pigmentation was observed for any of these bacterial strains.

Therefore only *Erwinia carotovora* subspecies *carotovora* (*Ecc*) was capable of heterologous expression of prodigiosin from cosmid pNRT104. However, there were differences in the ability to express pigment between different *Ecc* strains, with 9 out of the 36 *Ecc* strains tested failing to express pigment.

The *Ecc* colonies that were able to express prodigiosin (figures 3.4 a-e) revealed an unexpected level of variation in the expression of pigment, many exhibiting different pigmentation patterns within the colony. *Ecc* strain SCRI174 for example (figure 3.4a) exhibited a very intense level of pigmentation with some of the pigment leaching into the surrounding media. The SCRI144 colonies transformed with pNRT104 however exhibited a very low level of pigmentation and there appeared to be a defined area of expression in the centre with very low levels of expression throughout the rest of the colony. There were two colonies with sectors in which pigment expression had been abolished.

Figure 3.4c is interesting because it shows sectoring of a colony and reduced pigment variants. SCRI155-pNRT104 colonies (figure 3.4d) exhibited more intense pigmentation in the centre of the colony fading towards the edge, with a cessation in

TABLE 3.1

<i>Ecc</i> SCRI strains	Prodigiosin phenotype with pNRT104	<i>Ecc</i> SCRI strains	Prodigiosin phenotype with pNRT104
101	white	126	white
102	white	127	red
103	red	130	red
106	white	132	red
109	red	135	red
112	red	139	white
113	red	144	red
114	red	149	white
115	white	152	red
116	red	155	red
117	red	166	red
118	red	169	red
120	white	171	red
121	red	172	red
122	red	174	red
123	red	192	red
124	red	193	red
125	red	198	white

Table 3.1 Expression of prodigiosin by various *Ecc* strains transformed with cosmid pNRT104

Key:
white - failure to express visible levels of prodigiosin; red - visible levels of pigment production.

**FIGURE 3.4 GENE EXPRESSION IN THE BACTERIAL COLONY:
VARIOUS *Ecc* STRAINS TRANSFORMED WITH COSMID pNRT104**

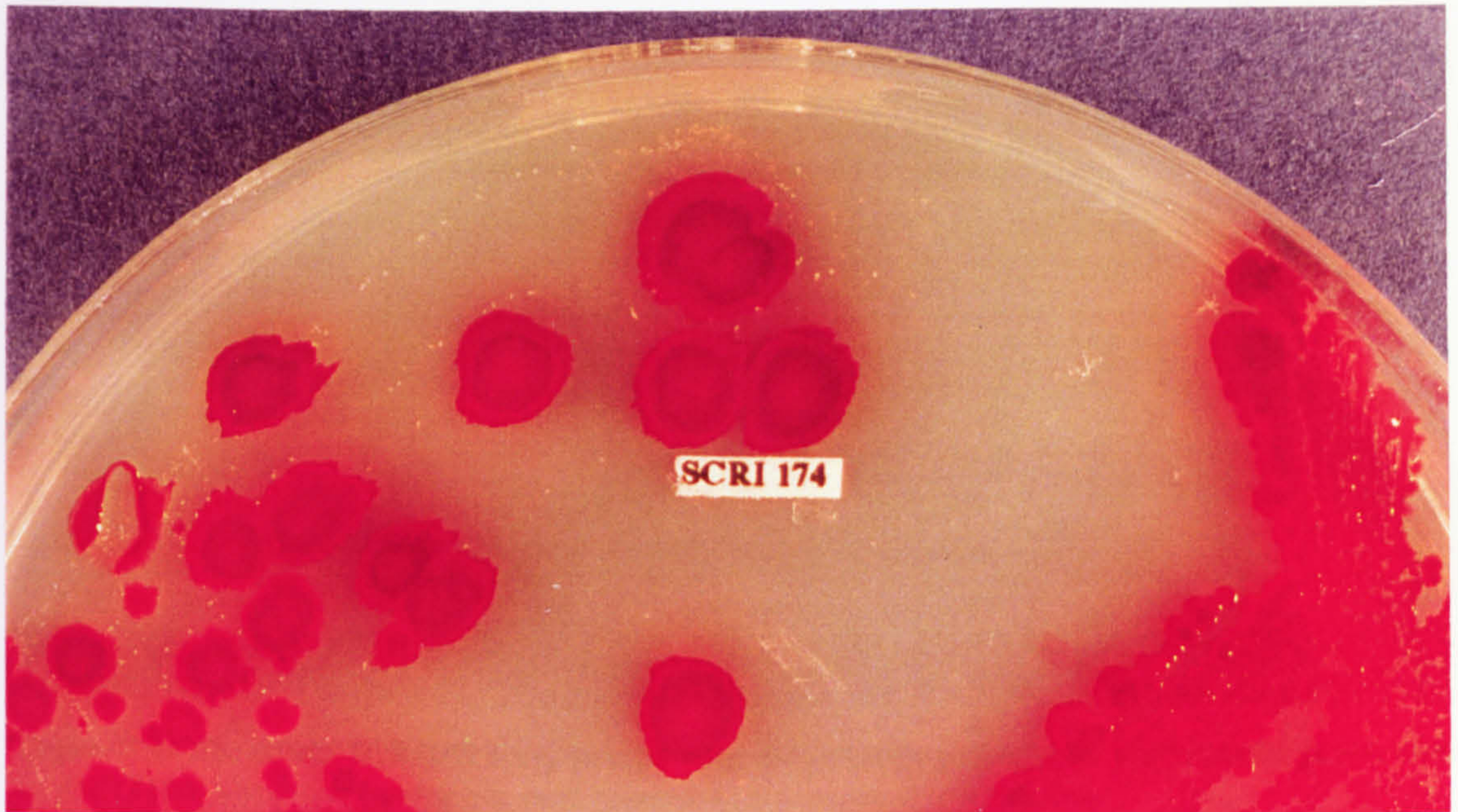


Figure 3.4a *Ecc* strain SCRI174 transformed with the putative prodigiosin biosynthetic genes, cloned in pNRT104.

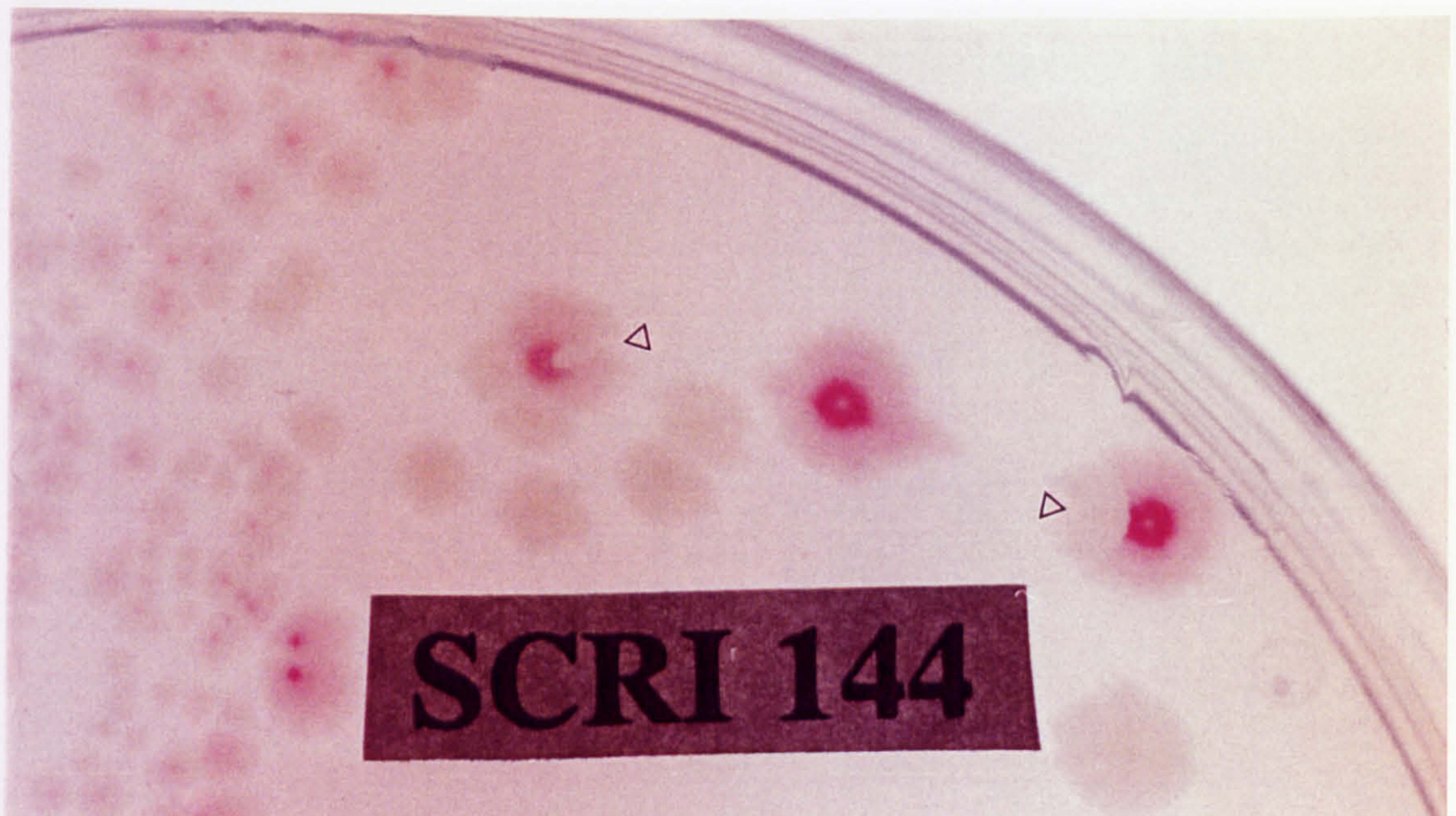


Figure 3.4b *Ecc* strain SCRI144 transformed with the putative prodigiosin biosynthetic genes, cloned in pNRT104. The colony sectors have been marked with open arrow heads.

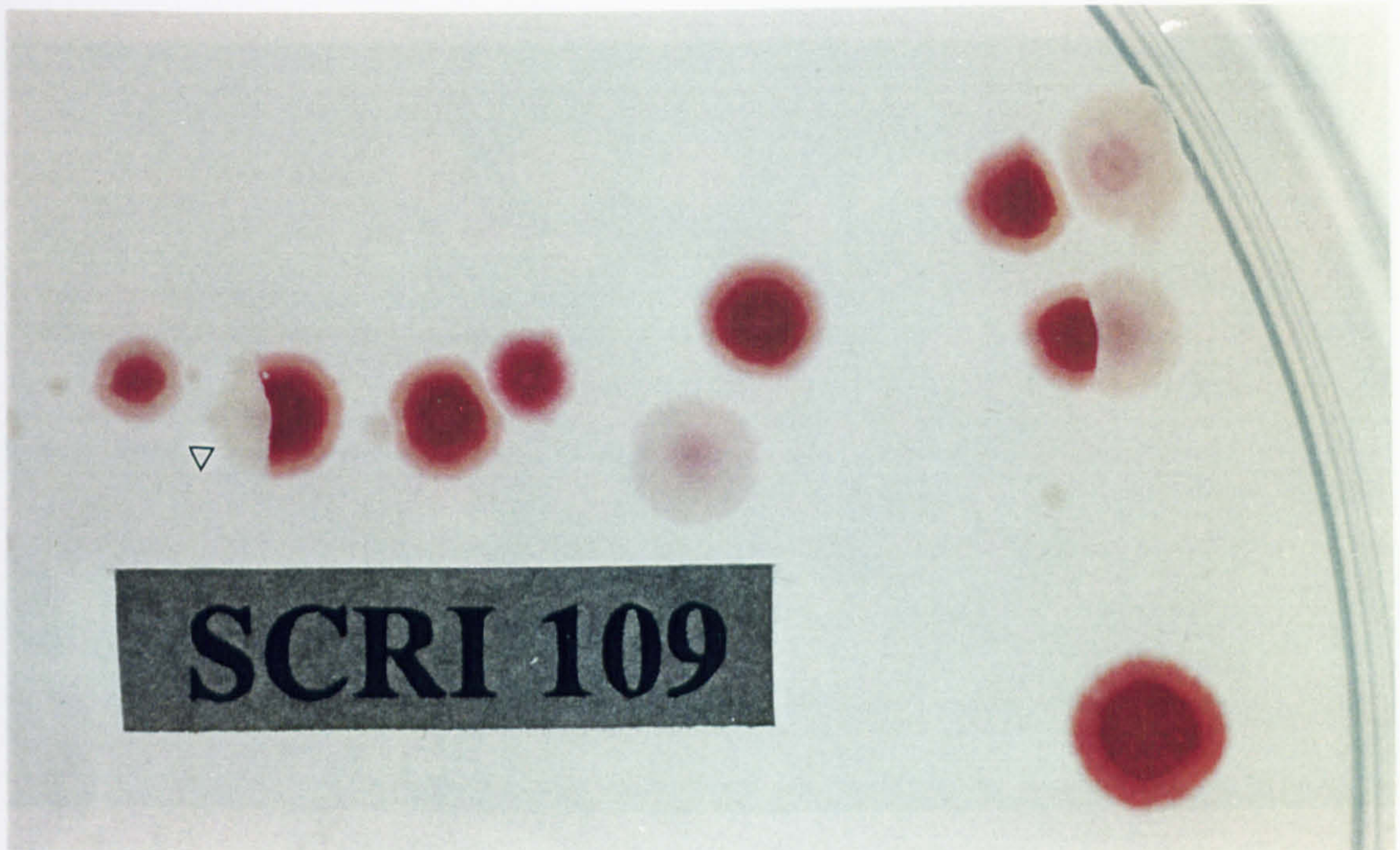


Figure 3.4c *Ecc* strain SCRI109 transformed with the putative prodigiosin biosynthetic genes, cloned in pNRT104. The colony sector has been marked with an open arrow head.

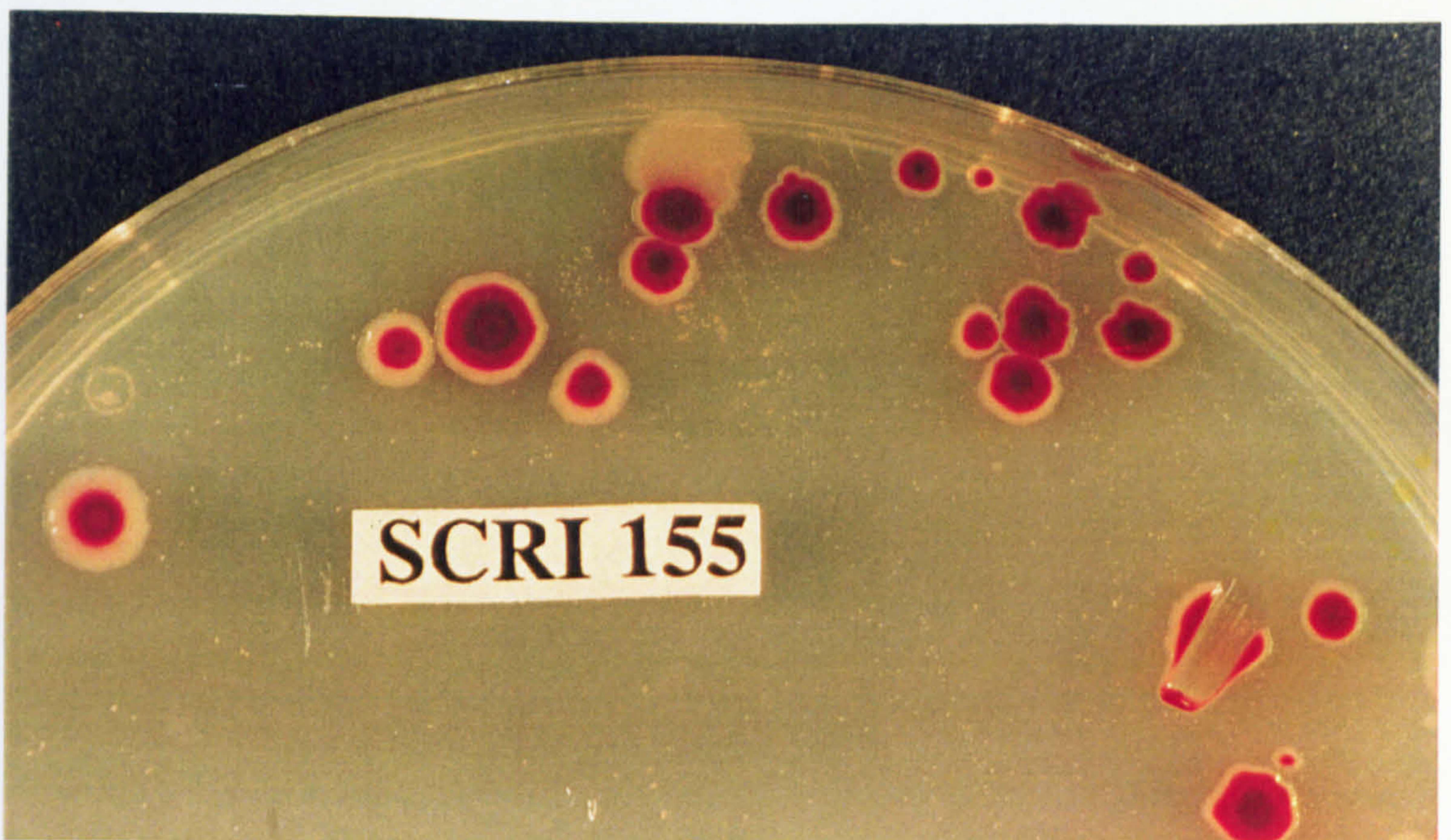


Figure 3.4d *Ecc* strain SCRI155 transformed with the putative prodigiosin biosynthetic genes, cloned in pNRT104

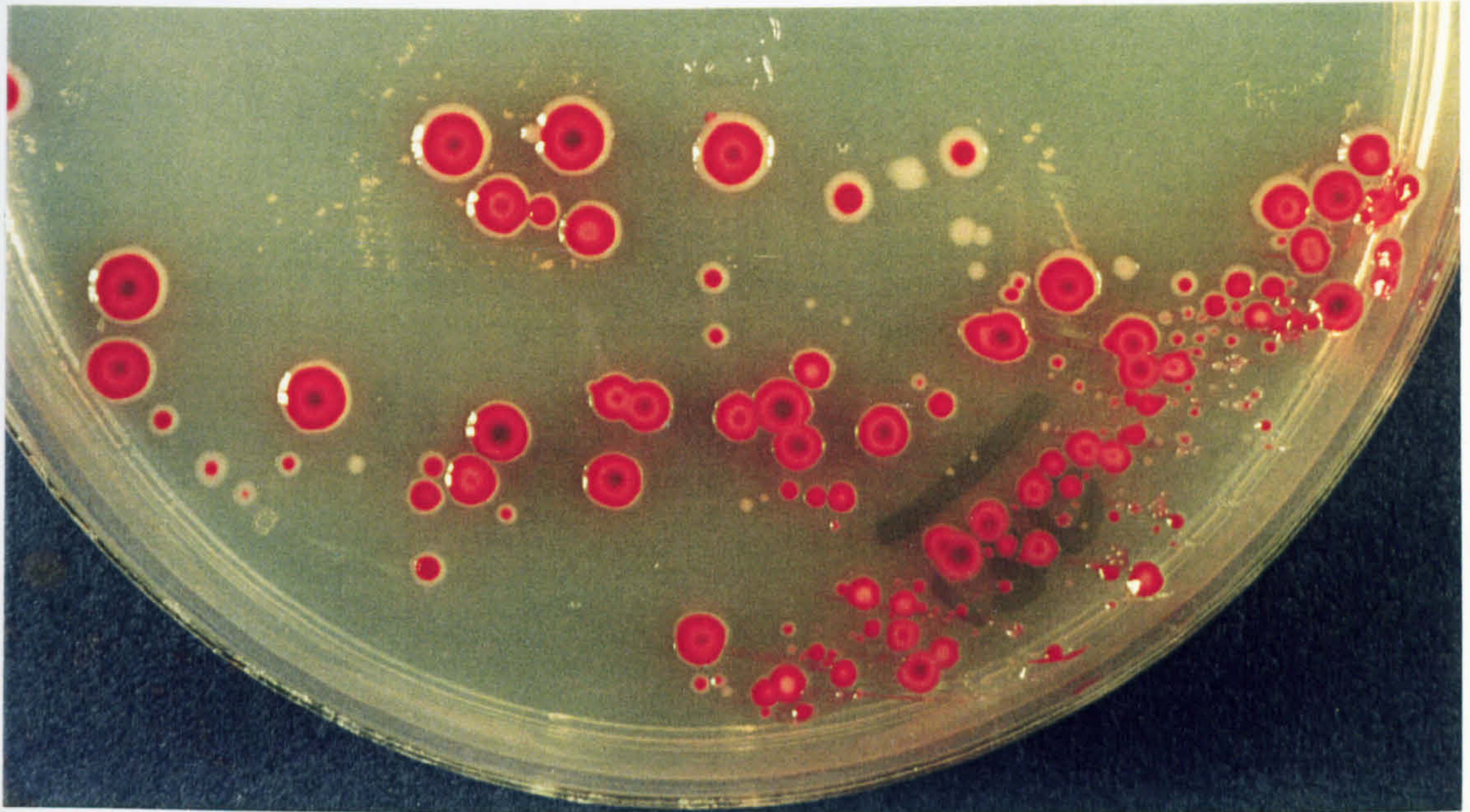


Figure 3.4e *Ecc* strain SCRI130 transformed with the putative prodigiosin biosynthetic genes, cloned in pNRT104.

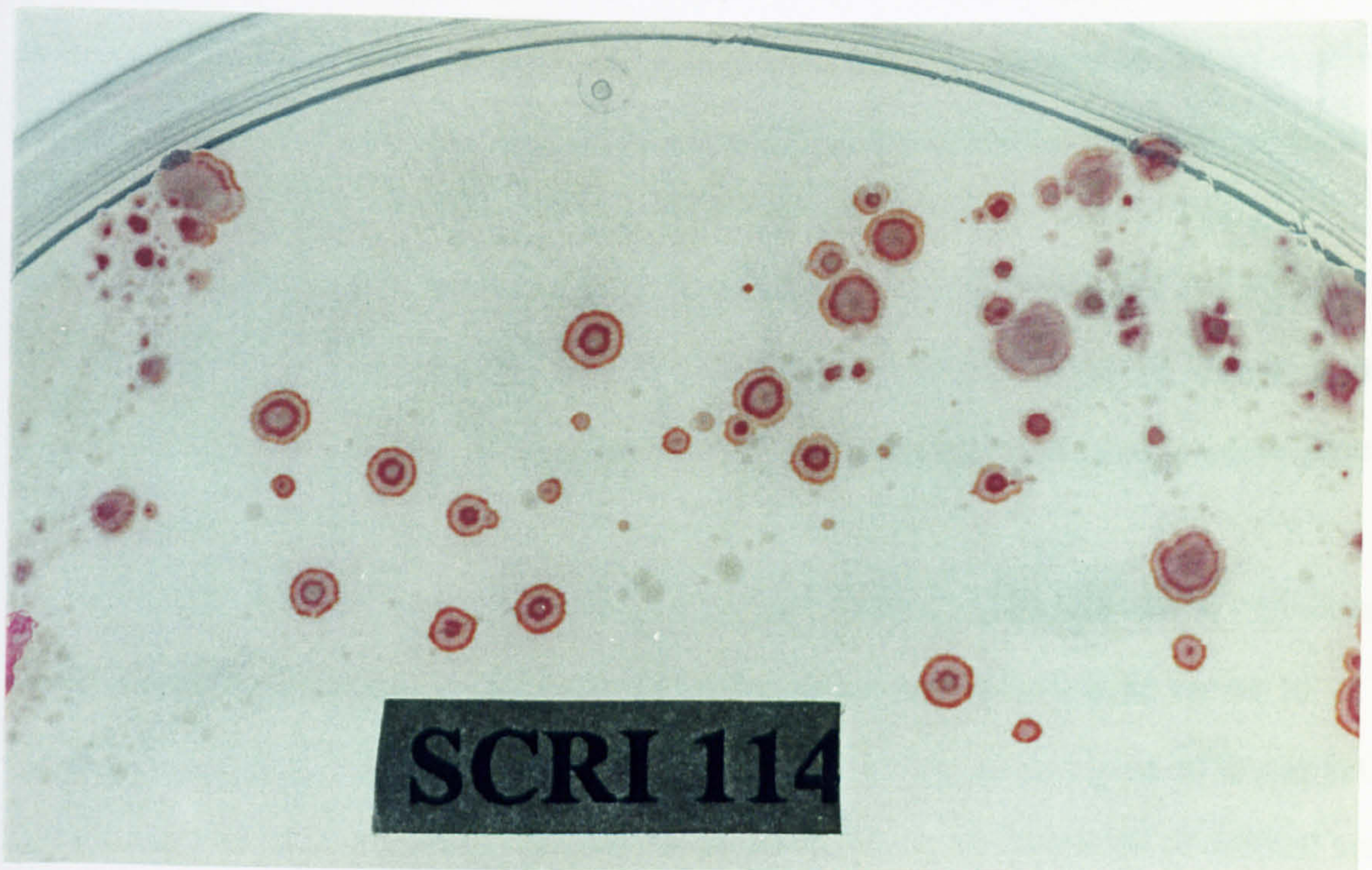


Figure 3.4f *Ecc* strain SCRI114 transformed with the putative prodigiosin biosynthetic genes, cloned in pNRT104

pigment production at the periphery of the colony. Figure 3.4e shows that SCRI130 colonies transformed with pNRT104, display a colony pigmentation that was 'fisheyed', having alternate concentric rings of high and low pigment expression, again with a distinct cut off in expression at the edge of the colony. SCRI114-pNRT104 colonies appeared to be the reverse of SCRI130-pNRT104, reminiscent of a rosette but with finer more discrete bands of pigment expression than exhibited by SCRI130-pNRT104 (figure 3.4f).

3.2 CONCLUSIONS

The pigment extracted from *Ecc* strain ATCC39048 transformed with pNRT104 was prodigiosin. It displayed the characteristic absorption maxima under acid and alkaline conditions. Of the bacteria tested, only *Ecc* strains could express prodigiosin heterologously. However the ability to express pigment was not ubiquitous within *Ecc* strains with 25% of them unable to express pigment. The addition of OHHL to *E.coli* and all the other bacteria transformed with pNRT104, which do not naturally produce autoinducer (table 3.2), failed to induce the production of pigment and so is taken to indicate that an additional factor(s), lacking in these bacteria, is needed for the synthesis of this secondary metabolite. The apparent inability to reconstitute pigment production in *E.coli* is consistent with the fact that the *pig* genes have never been cloned as a functional unit before in this bacterium. Alternatively, it is possible that the expression of these genes in *E.coli* is very poor. This has been previously report by Yanagida *et al.*, (1986) where it was found that the *Serratia* promoter for the serine protease gene was inoperative in *E.coli*.

The inability of some *Ecc* strains to express prodigiosin could not be explained in terms of OHHL expression, because all the *Ecc* strains tested have been shown to produce an autoinducer (Holden, 1996), consistent with the involvement of an, as yet, undefined factor(s). The banding patterns however betray the positions of defined areas of gene expression within the bacterial colony. The idea that a bacterial colony is not

just an amorphous mass of cells but a dynamic and highly differentiated system has been examined before. In a review by Shapiro, (1988) two features of growth within a bacterial colony became apparent: as the colony grows and it expands outwards, the apparent pattern of growth, or equally gene expression, consists of both concentric and radial elements. The radial elements or sectors consist of cells descended from common ancestry which differ genetically from other cells within the same colony which fall outside the sector. The concentric rings define areas where cells share common properties with all the cells at similar positions within the colony. There is evidence for both temporal and spatial factors which determine the patterns within the cell. Shapiro, (1988) highlighted that one clue to the reason for these concentric rings can be seen at the point at which concentric and radial elements overlap. Some radial elements are formed because cells within them have a higher growth rate. As a concentric ring passes through this sector the ring is stretched outwards, suggesting that the rings are formed at a specific time, the same for all the cells except at that time the faster growing cells are further away from the colony centre.

Spatial factors have been recorded in *Bacillus subtilis*. Differences in the colony density on an agar plate affected the time at which particular genes were expressed (Salhi and Mendelson, 1993). Differences in nutrient concentration and whether a colony was grown vertically or horizontally on an agar block were also factors which affected gene expression within a *Bacillus* colony. OHHL is known to play a role in the expression of prodigiosin in *Ecc*. It is possible that like the *S.marcescens car* genes, which are thought to be regulated by an OHHL-independent CarR protein (Cox, 1995), the *pig* genes are also regulated by a *luxR* homologue- '*pigR*'. In *Ecc* the *pig* genes may fall under the control of an undefined transcriptional activator, which is OHHL dependent in this genetic background. This would explain the requirement for OHHL for the expression of prodigiosin in *Ecc*. However, it is evident from the defined patterns of pigmentation that, because OHHL is diffusible, it is unlikely to be the only regulatory input. Other regulatory mechanisms, responding to conditions (such as nutrient

concentration gradients) within the colony microenvironment may override the induction by OHHL. Whatever the reason for the differences in the pigment ‘morphologies’ of the colonies, my results have revealed some dramatic differences in gene expression within the bacterial colony, which, because the patterns are so regular, must be tightly controlled.

The colonies in figure 3.4 (a-e) demonstrate both features described by Shapiro, (1988). Figure 3.4b shows a sector or radial element, judging from its size a spontaneous diminution in pigment production must have occurred early in the colony development. This reduced level of pigment production in several other colonies is comparable in intensity to the sector. Therefore this may indicate that the prodigiosin is toxic to the *Ecc* cells imposing a negative selection on the cells. Toxic effects of over expression of undecylprodigiosin, closely related to prodigiosin, have been reported previously (Malpartida *et al.*, 1990). If expression of prodigiosin in *Ecc* strain SCRI174 has caused the cells to lyse it may also explain why the agar in figure 3.4a was stained with pigment.

What is clear from these data is that prodigiosin expression in *Ecc* is regulated by a system which is exquisitely sensitive to unknown stimuli which could be concentration gradients or perhaps more temporal factors. This study has conclusively demonstrated that OHHL was not sufficient to allow the expression of prodigiosin in *Ecc* and may require multiple factors. More research would be required to determine what these additional factors are. However the results of this chapter although interesting have generated more questions than answers. It was difficult to see at this stage how continuing with this line of research would further the understanding of the regulation of antibiotic and pigment in *Serratia* which was the focus of this project. As a result the majority of the work detailed in the following chapters of this thesis concentrates on the isolation of *S.marcescens rap* gene(s) proposed to be encoded on pNRT300. Nevertheless this stunning variation of patterning of pigment expression in

recombinant *Ecc* strains warrants further study, perhaps as a novel project on intercellular communication in bacteria.

*Molecular Genetic investigation of
pNRT300,
the Rap complementing cosmid*

4.0 PREFACE

As discussed in the previous chapter pNRT300 was cloned by its ability to complement the antibiotic and pigment production defect in the Rap mutants NT2 and NT5. The cosmid pNRT300 was based on the low copy vector pSF6 (~ 1-3 copies per chromosome; Selveraij *et al.*, 1984) and the library was constructed such that each cosmid contained between 25-35kb of chromosomal insert (section 3.0).

To isolate the gene responsible for complementation of the Rap phenotype it was necessary to subclone the chromosomal insert to a manageable size for sequencing. Subcloning of pNRT300 was achieved by cloning various restriction enzyme fragments from pNRT300 and testing them for the ability to complement NT5.

4.1 SUBCLONING OF THE COSMID pNRT300

pNRT300 was cut with various restriction enzymes which had six base pair recognition sites, in order to cut the cosmid into a small number of large fragments. These restriction fragments were then ligated back into the pSF6 vector cut with the same enzyme. *E.coli* strain MC1061 was transformed by electroporation, with the ligation products, and transformants selected on NBA containing spectinomycin (50µg/ml). To test for complementation, NT5 was transformed, with each of the plasmid constructs, by electroporation and transformants selected on NBA plates containing spectinomycin. It was immediately obvious when NT5 had been complemented for the pigment phenotype. To test for carbapenem production transformants were then picked onto *E.coli* ESS bioassay plates.

In these complementation studies a ~15-20kb *Bam*HI (pNRT3200) and a ~7.5kb *Hind*III (pNRT370) restriction fragment was found to complement NT5 to Car⁺, Pig⁺ (data not shown). The smallest construct, pNRT370, was chosen for further analysis and was restriction mapped in preparation for further subcloning.

4.2 RESTRICTION MAPPING pNRT370

DNA was cut with restriction enzymes *Bam*HI, *Eco*RI, *Pst*I, *Sal*I, *Xba*I which all have unique cleavage sites in pSF6. These digests were then run on an agarose gel overnight to allow maximum separation of the restriction fragments (figure 4.1). To overcome some of the inaccuracies of estimating the size of restriction fragments from agarose gels, a plot of band size against distance was drawn using the 1kb BRL (section 2.2.8) commercial ladder as the standard (data not shown). Restriction fragment size(s) for single and double enzyme digests was then estimated and are recorded in table 4.1

TABLE 4.1

Gel Lane	Restriction enzyme(s) used	N ^o Restriction fragments	Restriction fragments generated (approx size*, given in kb)
2.	<i>Xba</i> I	1	19.6
3.	<i>Pst</i> I	2	5.8, 13.8
4.	<i>Eco</i> RI	2	1.8, 17.8
5.	<i>Sal</i> I	2	8.2, 11.4
6.	<i>Bam</i> HI	1	19.6
7.	<i>Xba</i> I \ <i>Pst</i> I	4	3.1 ^a , 5.6, 8.7 ^a , 14.0
8.	<i>Xba</i> I \ <i>Eco</i> RI	-	- ^b
9.	<i>Xba</i> I \ <i>Sal</i> I	3	3.6, 4.5, 11.5
10.	<i>Xba</i> I \ <i>Bam</i> HI	2	3.9, 15.7
11.	<i>Pst</i> I \ <i>Eco</i> RI	4	0.5, 1.4, 5.7, 12.0
12.	<i>Pst</i> I \ <i>Sal</i> I	4	1.1, 3.5, 4.7, 10.3
13.	<i>Pst</i> I \ <i>Bam</i> HI	3	3.7, 5.8, 10.1
14.	<i>Eco</i> RI \ <i>Sal</i> I	4	1.8, 2.1, 4.4, 11.3
15.	<i>Eco</i> RI \ <i>Bam</i> HI	3	1.8, 2.3, 15.5
16.	<i>Sal</i> I \ <i>Bam</i> HI	3	0.2 ^c , 8.2, 11.2

Table 4.1 Restriction fragments generated from single and multiple enzyme digests of pNRT370

Key:	<p>*-Restriction fragment size with an accuracy of approximately +/- 100bp.</p> <p>a-Restriction fragments appear to be the result of either partial enzyme digestion or star activity.</p> <p>b-These bands do not match the predicted restriction map based on the other digests and are thought to be due to experimental error.</p> <p>c-This fragment is a predicted internal vector fragment but was not visible on the agarose gel.</p>
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FIGURE 4.1

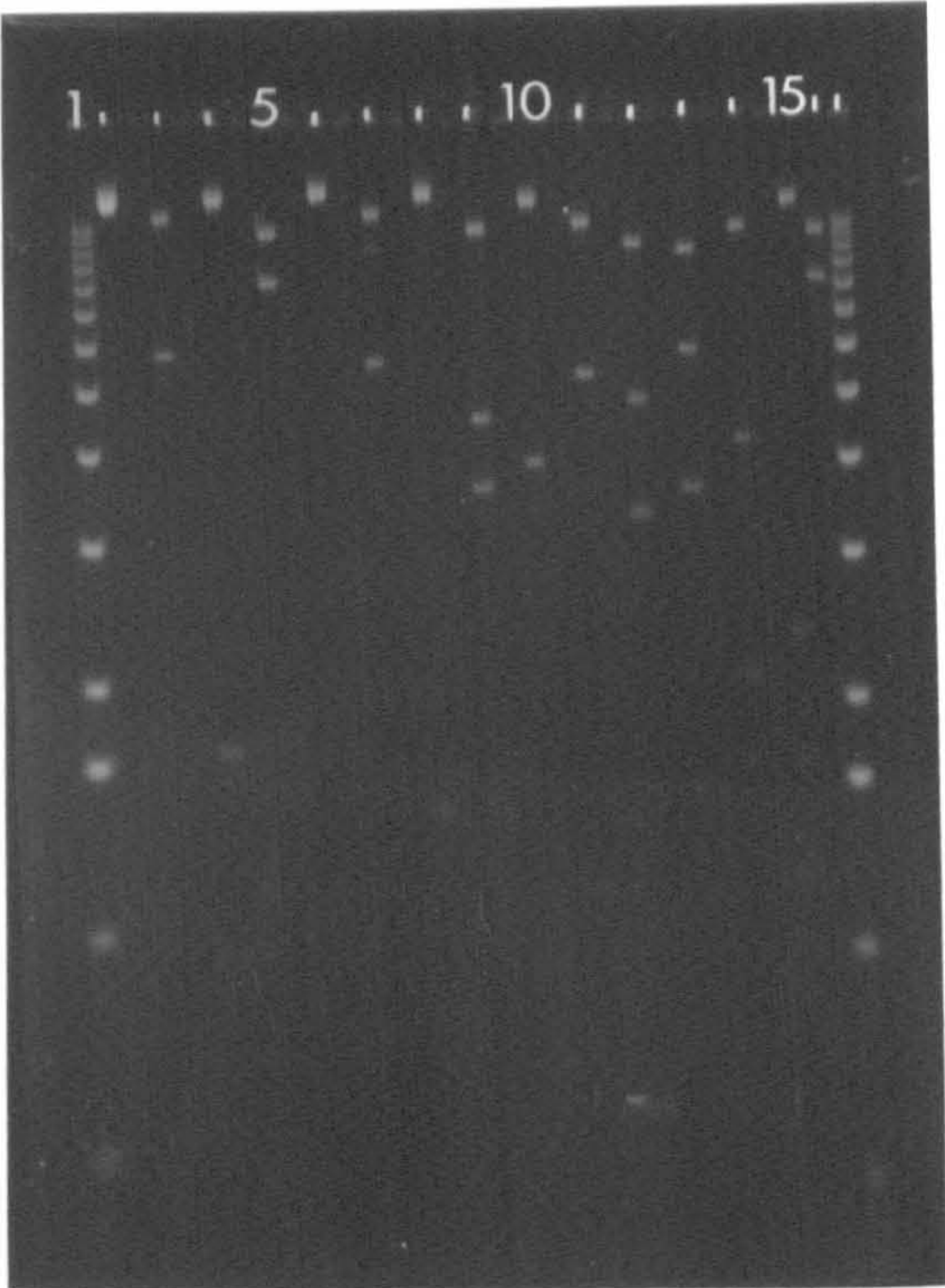


Figure 4.1 Restriction mapping pNRT370: An agarose gel showing single and double restriction enzyme digests of pNRT370

Key:					
Lane	Enzyme	Lane	Enzyme(s)	Lane	Enzyme(s)
1	1kb Ladder	7	<i>Xba</i> I/ <i>Sal</i> I	13	<i>Pst</i> I/ <i>Bam</i> HI
2	<i>Xba</i> I	8	<i>Xba</i> I/ <i>Eco</i> RI	14	<i>Eco</i> RI/ <i>Sal</i> I
3	<i>Pst</i> I	9	<i>Xba</i> I/ <i>Sal</i> I	15	<i>Eco</i> RI/ <i>Bam</i> HI
4	<i>Eco</i> RI	10	<i>Xba</i> I/ <i>Bam</i> HI	16	<i>Sal</i> I/ <i>Bam</i> HI
5	<i>Sal</i> I	11	<i>Pst</i> I/ <i>Eco</i> RI	17	1kb Ladder
6	<i>Bam</i> HI	12	<i>Pst</i> I/ <i>Sal</i> I		

4.2.1 ANALYSIS OF RESULTS FOR RESTRICTION MAPPING pNRT370

4.2.2 SINGLE ENZYME DIGESTS

Of the enzymes used *Xba*I and *Bam*HI did not cut in the insert and *Pst*I, *Eco*RI and *Sal*I were found to cut once (figure 4.1, table 4.1). Figure 4.2 illustrates the method used to position the cleavage sites of *Pst*I, *Eco*RI and *Sal*I within the insert and thereby construct a restriction map. Details of the individual digest are given below:-

***Pst*I:-** The 5.8 kb band placed the *Pst*I site at the right hand side of the chromosomal insert. Subtracting the distance from the vector *Pst*I site to the cloning site (~0.2 kb) located the *Pst*I site approximately 1.9 kb from the right and 5.6 kb from the left hand end of the chromosomal insert.

***Eco*RI:-** The gel clearly showed two bands; one of 1.8 kb and another of 17.8 kb. This result located the cleavage site 1.8 kb from the vector *Eco*RI site and approximately 0.1 kb from the *Hind*III site (figure 4.3).

***Sal*I:-** Similarly, this digest gave two bands; the important one being the 8.2 kb band which placed the *Sal*I site 4.5 kb from the left end and 3.0 kb from the right end of the 7.5 kb *Hind*III subclone (figure 4.3).

4.2.3 DOUBLE ENZYME DIGESTS

The predicted restriction map made from the single digest analysis (figure 4.3) was confirmed by studying the banding pattern for the multiple restriction enzyme digests (table 4.1).

4.2.4 ADDITIONAL RESTRICTION SITES

In a similar manner to that already described the *Eco*RV enzyme cleavage sites were mapped (data not shown). This enzyme did not digest in the vector but did digest three times in the 7.5 kb chromosomal insert giving three fragments of 3.9 kb, 1.4 kb and a 14.3 kb fragment containing 12.1 kb of pSF6. In this instance the mapped *Sal*I and *Pst*I sites in the chromosomal insert were used as reference points. Double digests

FIGURE 4.3

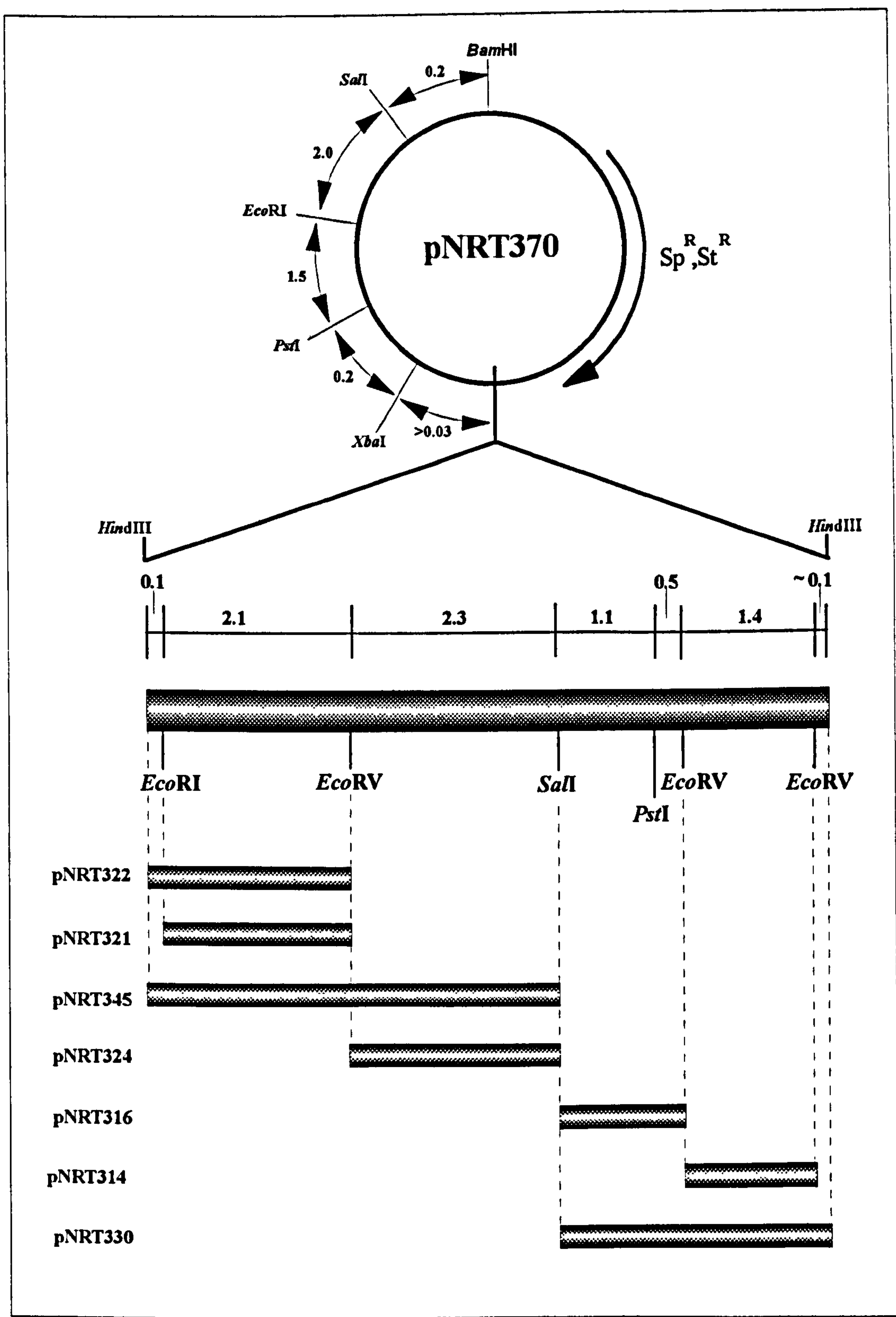


Figure 4.3 The predicted restriction map of pNRT370. The subclones generated from pNRT370 as a result of this map are drawn below, with the names they were designated down the side. All sizes are approximate and in kilobases. (Key: Sp^R - spectinomycin resistance; St^R - streptomycin resistance)

were performed with either *EcoRV* and *SalI* or *EcoRV* and *PstI*. The predicted restriction enzyme map for pNRT370 containing the *SalI*, *PstI*, *EcoRI* and *EcoRV* sites is illustrated in figure 4.3. This restriction map was subsequently used in the cloning of various fragments from pNRT370 with a view to finding the smallest subclone that could complement NT5.

4.3 SUBCLONING OF pNRT370

Using the restriction map shown in figure 4.3, the 7.5 kb insert of pNRT370 was cut with a variety of restriction enzymes. The fragments generated were then ligated into cloning vector pBR325 (Bolivar *et al.*, 1977) cut with compatible enzymes. The ligations were then used to transform *E.coli* strain MC1061 and transformants selected on NBA containing chloramphenicol (2.5µg/ml).

Cloning vector pBR325 was chosen because it has a higher copy number (~25 copies \ chromosome) than pSF6 (1-2 copies \ chromosome) and therefore would lead to the higher yield of plasmid DNA for subsequent cloning and sequencing. Also, the chloramphenicol antibiotic resistance could be used in conjunction with the antibiotic markers of other plasmids that were to be used in later experiments.

The fragments successfully subcloned are described in figure 4.3. Problems were encountered when cloning the 4.5 kb *HindIII-SalI* (pNRT345) and the 2.3 kb *EcoRV-SalI* (pNRT324) fragments. Extensive attempts to clone both of these fragments failed using *E.coli* strain MC1061 as a host. All recombinant plasmids derived from these ligations had undergone deletions of the insert. Transformants containing the 4.5kb *HindIII-SalI* fragment of pNRT370 were eventually isolated using *E.coli* strain DH1 as the recipient. However this construct (named pNRT345) made the cells grow very slowly; colonies remained small, even after prolonged incubation.

All the subclones successfully generated from pNRT370 (see figure 4.3) were used to electroporate NT5. Transformants were selected on NBA supplemented with chloramphenicol and then colonies were picked on to the carbapenem bioassay. Of the

subclones made, only the 4.5 kb *HindIII-SalI* fragment (pNRT345) complemented the Rap mutant NT5 to Car⁺, Pig⁺ (data not shown).

4.4 SUBCLONING OF pNRT345

It was obvious from the results described in section 4.3 that, because pNRT345 complemented NT5 and pNRT322 and pNRT321 did not, the putative complementing gene must either be on the 2.3 kb *EcoRV - SalI* (pNRT324) fragment, which had still to be cloned, or it spanned the *EcoRV* site (figure 4.3).

In an attempt to avoid possible deleterious copy number effects seen when cloning pNRT345, a lower copy number cloning vector pACYC184 (~15copies / chromosome. Chang and Cohen,(1978).see appendix) was cut with *EcoRV* and *SalI*, into which the 2.3 kb fragment was successfully subcloned, forming pNRT324. Even in this lower copy number vector cells containing pNRT324 exhibited unusual phenotypes. Cells viewed under electronmicroscopy appeared elongated (30 - 40 cell lengths) and, therefore, were possibly affected in cell division (figure 4.4).

NT5 was transformed with the pNRT324 subclone. The resulting transformants exhibited a Pig⁺ phenotype and subsequently, when tested on the ESS bioassay, were also found to be Car⁺. So the putative Rap complementing gene had been successfully subcloned down to this 2.3 kb chromosomal DNA fragment. The construct pNRT324 was considered of a suitable size for sequencing and was used for bulk preparation of the plasmid DNA in a Maxi prep which was run on a caesium chloride gradient.

4.5 DISCUSSION

The pSF6 based *S.marcescens* library clone pNRT300 was successfully subcloned, initially to a 7.5 kb *HindIII* fragment (pNRT370) then, on the production of a restriction map for pNRT370, down to a 2.3 kb *EcoRV-SalI* complementing subclone (pNRT324).

FIGURE 4.4

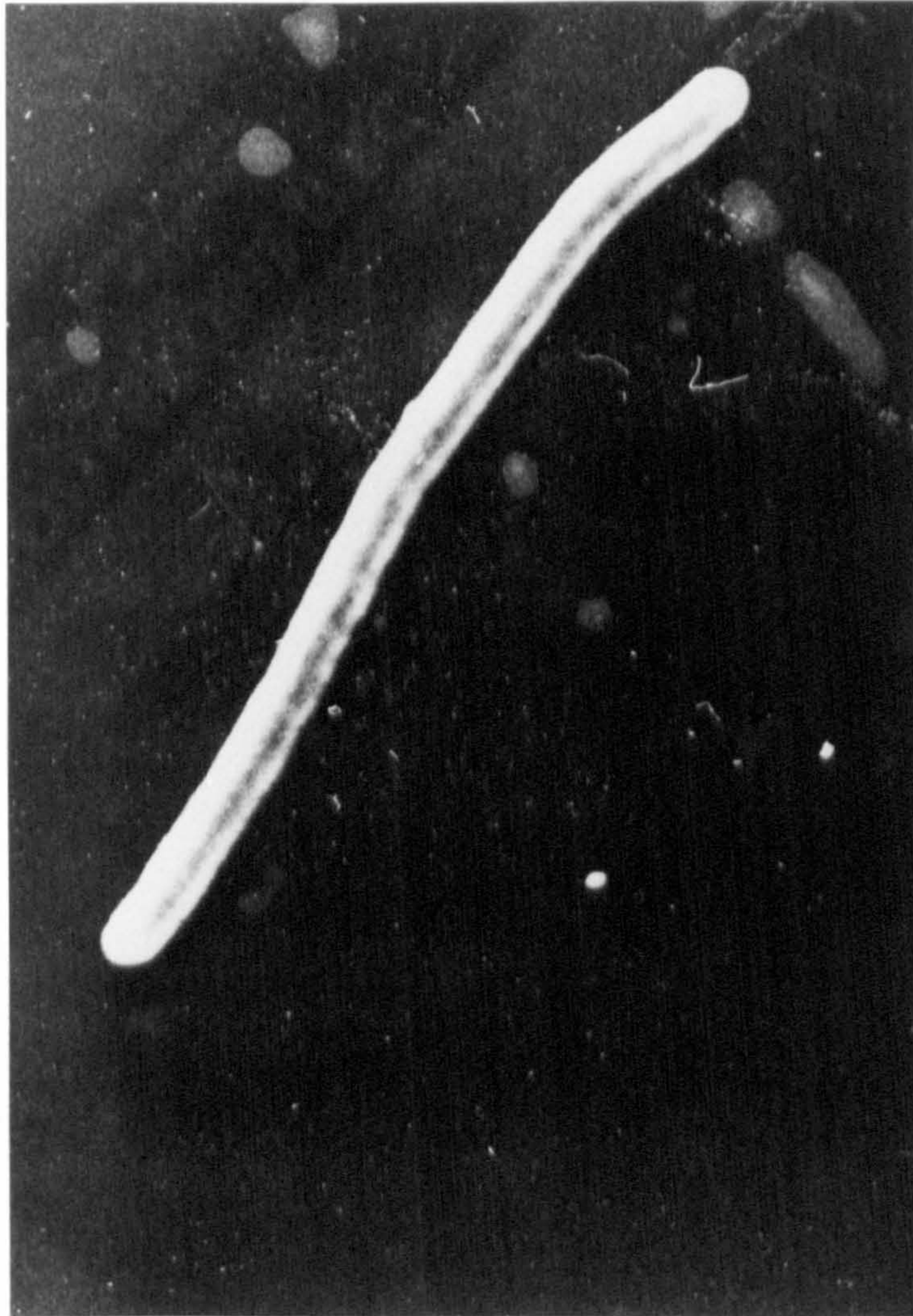


Figure 4.4 A scanning electron micrograph of *E. coli* strain DH1 transformed with pNRT324. Cells were treated as described in section 2.19. Cells appear elongated, reaching 29-30 cell lengths in size.

Difficulties were experienced in the cloning of the 4.5 kb *HindIII* - *SalI* fragment (pNRT345) and this was thought to be the result of a gene or a regulatory element on this insert which was deleterious to the host cell in multicopy. This may have acted as a negative selective pressure and only allowing survival of the recombinants in which recombination of the direct repeats (Prentki *et al.*, 1981) in the cloning vector pBR325 had occurred. This was also considered to be the likely reason why the majority of the subclones made from pNRT370 were stable in *E.coli* strain MC1061 in this vector whereas the 4.5 kb *HindIII*-*SalI* fragment was not. In addition I was unable to clone the 2.3 kb *EcoRV*-*SalI* into pBR325. The obvious reason for this is that this fragment was an internal subclone of pNRT345 and so the putative deleterious gene had been further localised to this fragment. The use of a lower copy vector (pACYC184) and *E.coli* strain DH1 (*recA1*) overcame the problems of cloning.

4 .6 SEQUENCE ANALYSIS OF pNRT324

The 2.3 kb *EcoRV*- *SalI* fragment of pNRT324 was sequenced using the M13 dideoxynucleotide chain termination method of Sanger *et al.*, (1977). The insert was cut from pNRT324 with *EcoRV* and *SalI* restriction endonucleases and prepared for random sequencing (section 2.8.1).

4.6.1 IDENTIFICATION OF OPEN READING FRAMES IN pNRT324

The nucleotide sequence derived from random sequencing was translated in six reading frames and the most likely open reading frames (Orfs) identified (section 2.9). The sequence revealed two complete Orfs and two partial Orfs. The sequence of pNRT324 and the putative Orfs are shown, together with the predicted translated protein sequence and the putative ribosome binding sites (Shine and Dalgarno, 1974), in figure 4.5. Putative promoter sequences have not been highlighted because none of those identified conformed well to the *E.coli* consensus (-35 -TTGACA and -10 - TATAAT; Hawley and McClure, 1983)

FIGURE 4.5

10 30 50
Sa11-----+-' orf1>>-----+-----+-----+-----+
GTCGACTCTTGGTGGGAGGTAATGTTGTCTTGCCCGAAGCATTTTTCTCATTGCTTGCCT
--ArgLeuLeuValGlyGlyAsnValValLeuProGluAlaPhePheSerLeuLeuAlaS
70 90 110
-----+-----+-----+-----+-----+
CTTTTTTACTAAAAAACGCAGTTTCGACAAACTGACTGGTATTTCCATAGTGACGAAA
erPheLeuLeuLysLysArgSerPheAspLysThrAspTrpTyrPheHisSerAspGluT
130 150 170
-----+-----+-----+-----+-----+
CTATTTTTATTGTTATTGTTAATCAATGCTTGCCAGCCGGGACGATCAACGCTTCAATAA
hrIlePheIleValIleValAsnGlnCysLeuProArgGlyThrIleAsnArgSerIleL
190 210 230
-----+-----+-----+-----+-----+
AAAGGTTCGATGAGGAGGAGGTCCCGTGGCTGAGCAACTTGAGCTTTTTCCTGTACCCAA
ysArgPheAspGluGluGluValProTrpLeuSerAsnLeuSerPhePheLeuTyrProI
250 270 290
-----+-----+-----+-----+-----+
TCCCTGTCGTGGTATCTGTCAGGCTGATGAGCGGGGTTTTTGCCGAGGGTGTTTTCGTAG
leProValValValSerValArgLeuMetSerGlyValPheAlaGluGlyValPheValA
310 330 350
-----+-----+-----+-----+-----+
CCGGTCGGAACGTTTTAGTTAGGGACAAATGAGCGATGTTTCAGAAACAGGATGTCTTACG
laGlyArgAsnValLeuValArgAspLysEnd
370 390 410
-----+-----+-----+-----+-----+
TCTTTGTCTGCGCAAAGGATGAAACGATTGCAGCGTGTTGAGAAAATTTCAATACCGCCGGA
430 450 470
-----+-----+-----+-----+-----+
ACCAGAGCAATCGAGTTTGTTTTAAGATAGGTCGTGATATTTTGTGTAATAAATTAAGTT
490 510 530
-----+-----+-----+-----+-----+
TAAGATCATTTTTAGGTAAC TATTATTGGTAGAATACTTATTGTATTTACAATAAATAT
550 570 590
-----+-----+-----+-----+-----+
ATATTTTTCGTTTTGTCTTATTTTCATTTTATAAATCAGAGTTGATTGCAATCCAGGGA
610 630 650
-----+-----+-----SD---+-- orf2>>-----+-----+-----+
TATAACTTAGCATGCTAATTATAAGGAGTAGTGATGGAATTGCCATTAGGATCTGATTTA
MetGluLeuProLeuGlySerAspLeu
670 690 710
-----+-----+-----+-----+-----+
GCCCCGTTAGTACGTGTTTGGCGCGCCTTGATAGACCACCGTTTGAAGCCGTTAGAACTT
AlaArgLeuValArgValTrpArgAlaLeuIleAspHisArgLeuLysProLeuGluLeu
730 750 770
-----+-----+-----+-----+-----+
ACACAGACGCACTGGGTGACGCTGCATAATATTCATGAACTGCCCCCAGGGCAGTCCCAA
ThrGlnThrHisTrpValThrLeuHisAsnIleHisGluLeuProProGlyGlnSerGln


```

      790              810              830
-----+-----+-----+-----+-----+-----+
ATTCAGTTGGCGAAGGCGATTGGTATTGAACAACCGTCATTGGTCCGCACTTTGGATCAG
IleGlnLeuAlaLysAlaIleGlyIleGluGlnProSerLeuValArgThrLeuAspGln

      850              870              890
-----+-----+-----+-----+-----+
CTTGAAGATAAGGGATTGATTACCAGACATATATGTGTACATGATCGCCGAGCTAAGCGC
LeuGluAspLysGlyLeuIleThrArgHisIleCysValHisAspArgArgAlaLysArg

      910              930              950
-----+-----+-----+-----+-----+
ATTATGCTGACAGATATGGCTGACCCGATCATTTCAGGCGGTTAATGATGTTATCGATCAA
IleMetLeuThrAspMetAlaAspProIleIleGlnAlaValAsnAspValIleAspGln

      970              990             1010
-----+-----+-----+-----+-----+
ACGCGCAGTGAAATATTAAATGGAATCACACCTGAAGAGGTTAGTGAATTAGCGACCATA
ThrArgSerGluIleLeuAsnGlyIleThrProGluGluValSerGluLeuAlaThrIle

     1030             1050             1070
-----+-----+-----+-----+-----+
ATTTCCAGGTTGGAAAGCAATATTCTATCGTTATATGAAATGCAGTCTTAATTTGTATGG
IleSerArgLeuGluSerAsnIleLeuSerLeuTyrGluMetGlnSerEnd

     1090             1110             1130
-----+-----+-----+-----+-----+
CACTTGAAAAATCTTGGTTTTTAAAAATTAAATTCTGCTGATTGTTAATTAAATTGCCATT

     1150             1170             1190
-----+-----+-----+-----+-----+
AACAGAGTGTTTTGCTGAAATTTAATTTTGCCGATTAATCACCCCTCGTTAGAGTGGTGA

     1210             1230             1250
-----+-----+-----+-----+-----+
TTAATCGATTTGCAATTAGCGAGGAGAAACAGTTACTGAACGGCCATCACTGACCATTGC
      EndArgProSerValThrValSerArgGlyAspSerValMetAla

     1270             1290             1310
-----+-----+-----+-----+-----+
GATTCTCTGACCAGCATAGAATTTGGTAGTACCTTGTTTTTGTACCACCATAATGGTATT
IleArgGlnGlyAlaTyrPheLysThrThrGlyGlnLysGlnValValMetIleThrAsn

     1330             1350             1370
-----+-----+-----+-----+-----+
GCCGTCATCTTTACGGATTTCCAGTTGAACACCTTGAACACGGTTTATCGCTCCCTCAAC
GlyAspAspLysArgIleGluLeuGlnValGlyGlnValArgAsnIleAlaGlyGluVal

     1390             1410             1430
-----+-----+-----+-----+-----+
ACTTTGCCCAGCGACACCACCTGCGACAGCACCGGCGGTGGCCAGGCTACGTCCGGT
SerGlnGlyAlaValGlyGlyAlaValAlaGlyAlaAlaThrAlaLeuSerArgGlyThr

     1450             1470             1490
-----+-----+-----+-----+-----+
TCCTCCACCAACAGTATTGCCAGGAAGCCTCCCAAGACTGCCCCGCCAAGAGCGCCAAT
GlyGlyGlyValThrAsnGlyLeuPheGlyGlyLeuValAlaGlyGlyLeuAlaGlyIle

```

```

      1510                      1530                      1550
-----+-----+-----+-----+-----+-----+
AATGTTATTCTCTTCGCCAGCCTGTATTTGTACTGGCCGTGTAGAAATAATTGTGCCGTA
IleAsnAsnGluGluGlyAlaGlnIleGlnValProArgThrSerIleIleThrGlyTyr

      1570                      1590                      1610
-----+-----+-----+-----+-----+-----+
AGTTACGGTTTGGACCTGTTTGGCATCAGATGCGCTGTAAACGTCTCCAGATAATGTACT
ThrValThrGlnValGlnLysAlaAspSerAlaSerTyrValAspGlySerLeuThrSer

      1630                      1650                      1670
-----+-----+-----+-----+-----+-----+
GGTATTTGCACAGCCAACCAAAGTGATACTTGCTAAAGTAATCACAAGAAAATACTTCAT
ThrAsnAlaCysGlyValLeuThrIleSerAlaLeuThrIleValLeuPheTyrLysMet
      ↑

      1690                      1710                      1730
<<orf3---+SD-----+-----+-----+-----+-----+
CATTTAAGGCTCCTGTAATGGCGATGTCGGACTGCGCTGCCCTTGTACCGACTAAATTAT
Met

      1750                      1770                      1790
-----+-----+-----+-----+-----+-----+
TTACATGCAGATTATGACGTGGACTAAAGCACCATTCCACTTTTTTACCTGATCACCAGG

      1810                      1830                      1850
-----+-----+-----+-----+-----+-----+
ATAAGATAATGCACTTAACTTTATAATAAGCCGACTATAAAGTTCACGTAAAGCCCCAT

      1870                      1890                      1910
-----+-----+-----+-----+-----+-----+
TTTTGTGAAAAGACATGTCAAATGGTTATTTTTTACAAAACACTGGCGACAACATAGAAAA

      1930                      1950                      1970
-----+-----+-----+-----+-----+-----+
AATGACAAAAGTCCACTAAGAATGTCGGTGAAATGCTATTTTTATCGGGTACATTTCTCA

      1990                      2010                      2030
-----+-----+-----+-----SD-----orf4'>>-----+
CCTGCCATTACTGTGTGATCCCCACGGAAAGTAAAGGTTAATAAGATGAAGTCAGGCAG
MetLysSerGlyAr

      2050                      2070                      2090
-----+-----+-----+-----+-----+-----+
ATATATTGGCGTGATGTCGGGAACCAAGTCTTGATGGTATTGATGTGGTGCTGGCCGCGAT
gTyrIleGlyValMetSerGlyThrSerLeuAspGlyIleAspValValLeuAlaAlaIl

      2110                      2130                      2150
-----+-----+-----+-----+-----+-----+
TGATGAATACACTGTAGCGCAGCAAGCCAGCTATTGCCATCCGATCCCACAGTCTATCAG
eAspGluTyrThrValAlaGlnGlnAlaSerTyrCysHisProIleProGlnSerIleAr

      2170                      2190                      2210
-----+-----+-----+-----+-----+-----+
GCTGGCTATTCTCAGCATGTGCCAGGGGCAACCGGTGACATTGTCGGCACTTGGGCATTT
gLeuAlaIleLeuSerMetCysGlnGlyGlnProValThrLeuSerAlaLeuGlyHisLe

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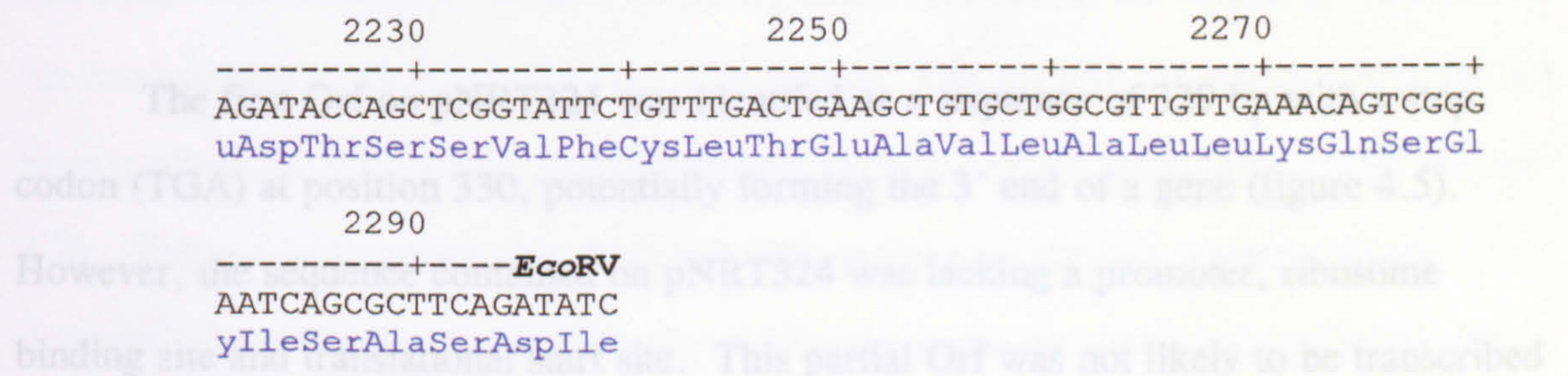



Figure 4.5 The 2299 nucleotide sequence of the *Sall*-*EcoRV* fragment of pNRT324. The protein translation of the sequence is written in blue below the nucleotide sequence. The initiation codons have been emboldened and the ribosome binding sites (red, SD- Shine and Dalgarno) are marked in coloured type. The termination codon is indicated by End in the protein translation below. The putative lipoprotein leader peptide cleavage site for Orf3 is marked with an arrow below the protein sequence (nucleotide position 1630).

FIGURE 4.6

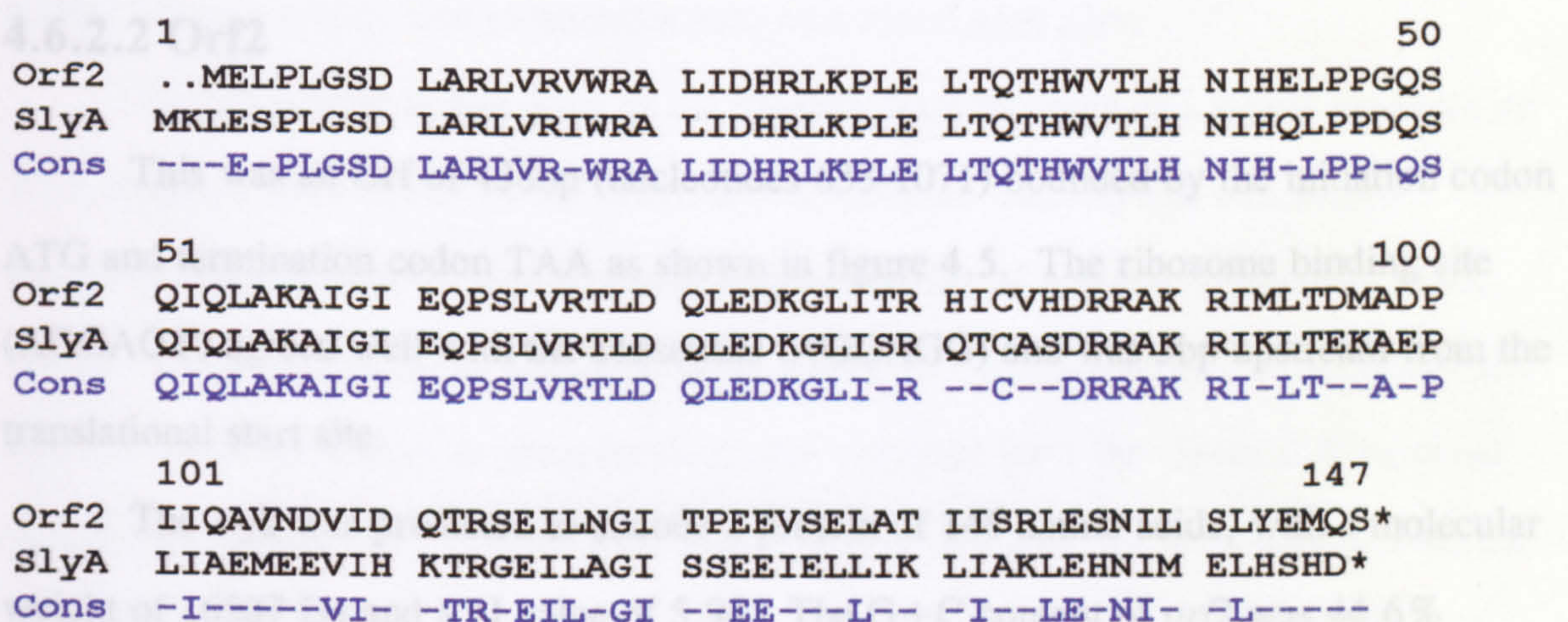


Figure 4.6 A lineup of the predicted product of Orf2 and SlyA
The pileup of Orf2 (*S. marcescens*, this study) and SlyA (*Salmonella typhimurium*; Libby *et al.* (1994); Accession number UO3824) was compiled using PILEUP of the UWGCG package. The consensus is written below (**Cons**) showing absolute amino acid conservation. The level of amino acid identity was 71%.

4.6.2 ANALYSIS OF THE OPEN READING FRAMES DETECTED IN THE SEQUENCE OF pNRT324

4.6.2.1 'Orf1

The first Orf on pNRT324 was identified as a sequence of 329 bp with a stop codon (TGA) at position 330, potentially forming the 3' end of a gene (figure 4.5). However, the sequence contained on pNRT324 was lacking a promoter, ribosome binding site and translational start site. This partial Orf was not likely to be transcribed by read through from plasmid based promoters either, because of the orientation the 2.3 kb insert had been cloned with respect to the plasmid based tetracycline promoter. (see the plasmid map for pNRT324 in the appendix).

The predicted truncated protein would be 109 amino acids long which, on searching the database, was found to share a very low level of homology with a number of proteins. This gene was not considered relevant to the search for the gene responsible for the regulation of antibiotic and pigment (the *rap* gene) in *Serratia* and will not be discussed further.

4.6.2.2 Orf2

This was an Orf of 438bp (nucleotides 633-1071) bounded by the initiation codon ATG and termination codon TAA as shown in figure 4.5. The ribosome binding site (AGGAGT) agreed well with the consensus (AGGAGG) and was 5bp upstream from the translational start site.

The *orf2* was predicted to encode a protein of 146 amino acids, with a molecular weight of 16507 Da and a pI value of 5.94. The G+C content of *orf2* was 44.6% which is low when compared with the average value for *Serratia marcescens* of ~58% (Grimont and Grimont, 1978). The protein retrieved from the data base with the highest level of homology with Orf2 was SlyA, a protein from *Salmonella typhimurium* (figure 4.6)

The pileup revealed a high level of amino acid identity between Orf2 and SlyA of 71%. These proteins shared a higher level of homology at the N-terminus with only 8 non conserved residues in the first 80 amino acids. However, the homology between the two proteins declined towards the C-terminus. This drop in the conservation did not greatly affect the hydropathy profiles of these proteins, which were very similar (figure 4.7). TMpred hydropathy plot predicted Orf2 to have no obvious transmembrane domains. In addition to the hydropathy profile the prediction made using PSORT for Orf2 and SlyA was that they were both cytoplasmic proteins consistent with the lack of an N-terminal signal sequence. The discovery that SlyA and the protein product of Orf2 were homologous formed a central theme in the remainder of this study and will be discussed in some detail below.

The SlyA protein was defined as a virulence factor because it was essential for the survival of *Salmonella* in peritoneal macrophages (Libby *et al.*, 1994). The *slyA* gene was isolated whilst looking for the haemolysin genes from *Salmonella*. A *Salmonella* cosmid library was transduced into *E.coli* and a clone containing *slyA* isolated from a colony that exhibited a halo on a blood agar plate.

The SlyA protein had none of the obvious features, such as a signal sequence or RTX motifs, characteristic of the previously discovered haemolysins and so was concluded to be a member of a completely novel family of haemolysins (Libby *et al.*, 1994). The lack of a signal sequence is not, however, uncharacteristic of haemolysins. HlyA a haemolysin from uropathogenic *E.coli*, does not have the classical N-terminal signal sequence and relies on the products of *hlyB,D* and *tolC* for its secretion from the cell (Welch, 1991).

A 680bp PCR product containing *slyA* had been used in a Southern blot against various chromosomal DNAs including 17 *Salmonella* isolates, 3 *Shigella* serotypes and 18 other pathogenic bacteria including *Yersinia*, *Proteus*, *Enterobacter* and *Serratia* species. Homologues were only detected in *Salmonella*, *Shigella* and enteroinvasive

FIGURE 4.7

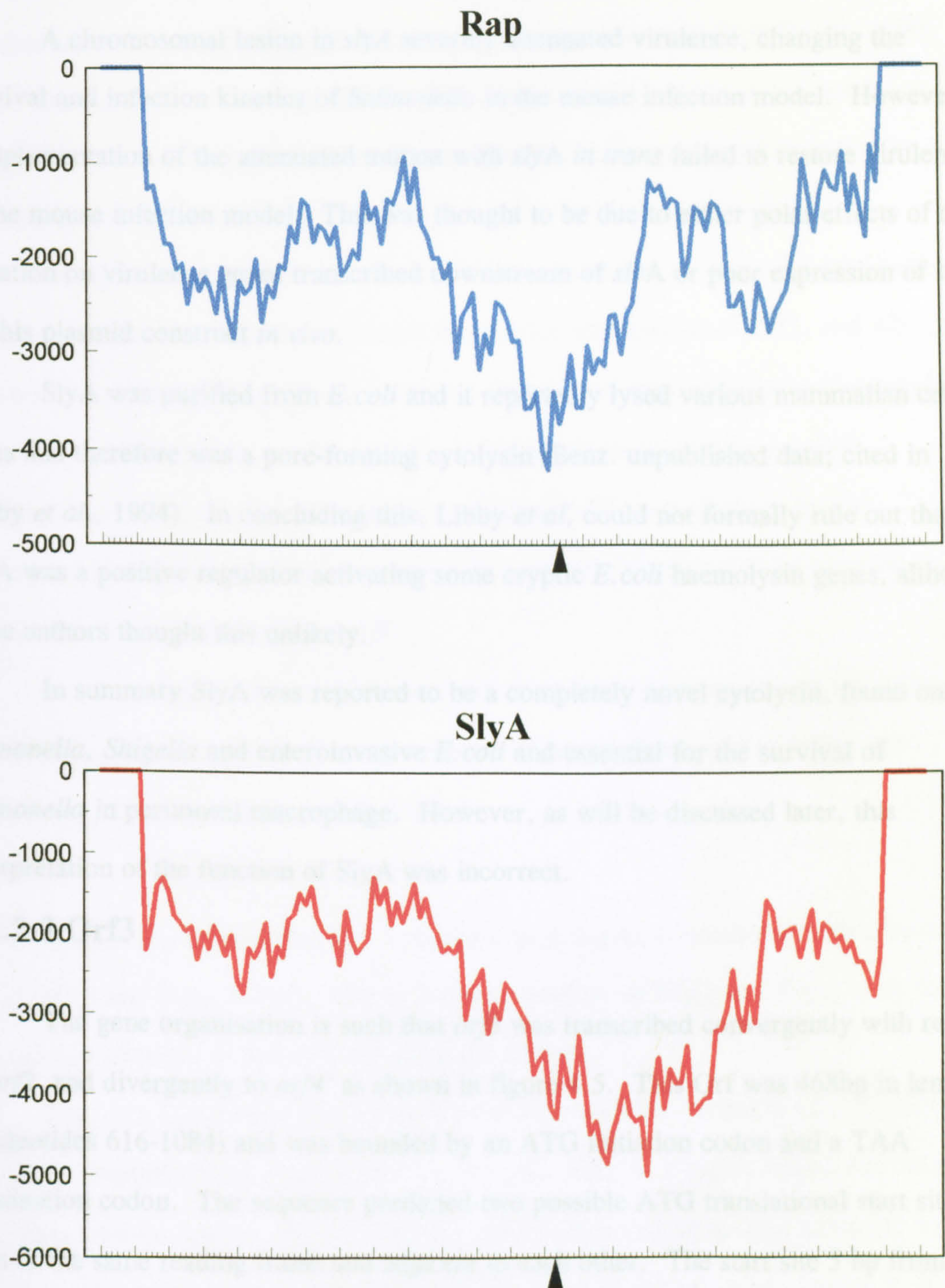


Figure 4.7 The predicted hydropathy profile of SlyA and the protein product of *orf2* drawn using TMpred. The window for this program was set at 17 residues and scores in excess of 500 are considered to be significant in representing a membrane spanning domain. The prediction made from these data was that there were no possible transmembrane domain for either of the two proteins displayed. The position of residue 80 is marked, at this point the identity between the two proteins declines.

E.coli (Libby *et al.*, 1994) consistent with the purported role of SlyA as a virulence factor in these invasive human and animal pathogens.

A chromosomal lesion in *slyA* severely attenuated virulence, changing the survival and infection kinetics of *Salmonella* in the mouse infection model. However, complementation of the attenuated mutant with *slyA in trans* failed to restore virulence in the mouse infection model. This was thought to be due to either polar effects of the mutation on virulence genes transcribed downstream of *slyA* or poor expression of *slyA* on this plasmid construct *in vivo*.

SlyA was purified from *E.coli* and it reportedly lysed various mammalian cell types and therefore was a pore-forming cytolysin (Benz. unpublished data; cited in Libby *et al.*, 1994). In concluding this, Libby *et al.*, could not formally rule out that SlyA was a positive regulator activating some cryptic *E.coli* haemolysin genes, although these authors thought this unlikely.

In summary SlyA was reported to be a completely novel cytolysin, found only in *Salmonella*, *Shigella* and enteroinvasive *E.coli* and essential for the survival of *Salmonella* in peritoneal macrophage. However, as will be discussed later, this interpretation of the function of SlyA was incorrect.

4.6.2.3 Orf3

The gene organisation is such that *orf3* was transcribed convergently with respect to *orf2* and divergently to *orf4'* as shown in figure 4.5. This Orf was 468bp in length (nucleotides 616-1084) and was bounded by an ATG initiation codon and a TAA termination codon. The sequence predicted two possible ATG translational start sites, both in the same reading frame and adjacent to each other. The start site 5 bp from the predicted ribosome binding site has been highlighted in figure 4.5. The predicted ribosome binding site (AGGAGC) fitted well with the consensus. The G+C content of the Orf was 48.6% which is low for *Serratia marcescens* with the average set at ~58% (Grimont and Grimont, 1978).

The *orf3* was predicted to encode a protein of 155 amino acids with a predicted molecular weight of 1554Da and a pI of 9.97. The first 17 amino acids of the N-terminus had all the characteristics of a lipoprotein signal peptide (Von Heijne, 1985; 1986). The proteolytic cleavage site for Orf3 was marked by the cysteine residue at position 18. The mature lipoprotein, after proteolytic cleavage, would therefore be 138 amino acids in length.

The signal peptide cleavage site for Orf3 is illustrated in figure 4.5 (at position 1632 in the sequence). The determinants for cellular localisation of Orf3, and other lipoproteins, are discussed in detail (section 6.2.2) and so they will not be discussed here. TMpred hydropathy profile analysis indicated there were three possible transmembrane helices. The first, between residues 6-22, was likely to be the lipoprotein signal peptide. The other two α -helices were predicted to be between residues 60-81 and 83-100 (figure 4.8).

Two homologous lipoproteins were retrieved from database searches for Orf 3, namely Pcp_{Ye} and Pcp_{Hi} from *Yersinia enterocolitica* and *Haemophilus influenzae* respectively, these are lined up in figure 4.9

The pileups showed that Orf 3 shared a high degree of amino acid identity with Pcp_{Ye} (71 %) and Pcp_{Hi} (47%). The hydropathy profiles of Pcp_{Hi} and Pcp_{Ye} were found to be almost identical to that of Orf3 (data not shown).

Pcp_{Hi} is a lipoprotein first isolated from *Haemophilus*, where it was co-purified with PAL (Peptidoglycan Associated Lipoprotein). PAL is a highly conserved outer membrane protein against which antibodies which conferred protective immunity to both typeable and non-typeable *Haemophilus* species could be raised. Even though it shared little homology with PAL, Pcp_{Ye} was co-precipitated with anti-PAL antisera, hence the nomenclature Pcp - PAL cross reacting protein (Deich *et al.*, 1988).

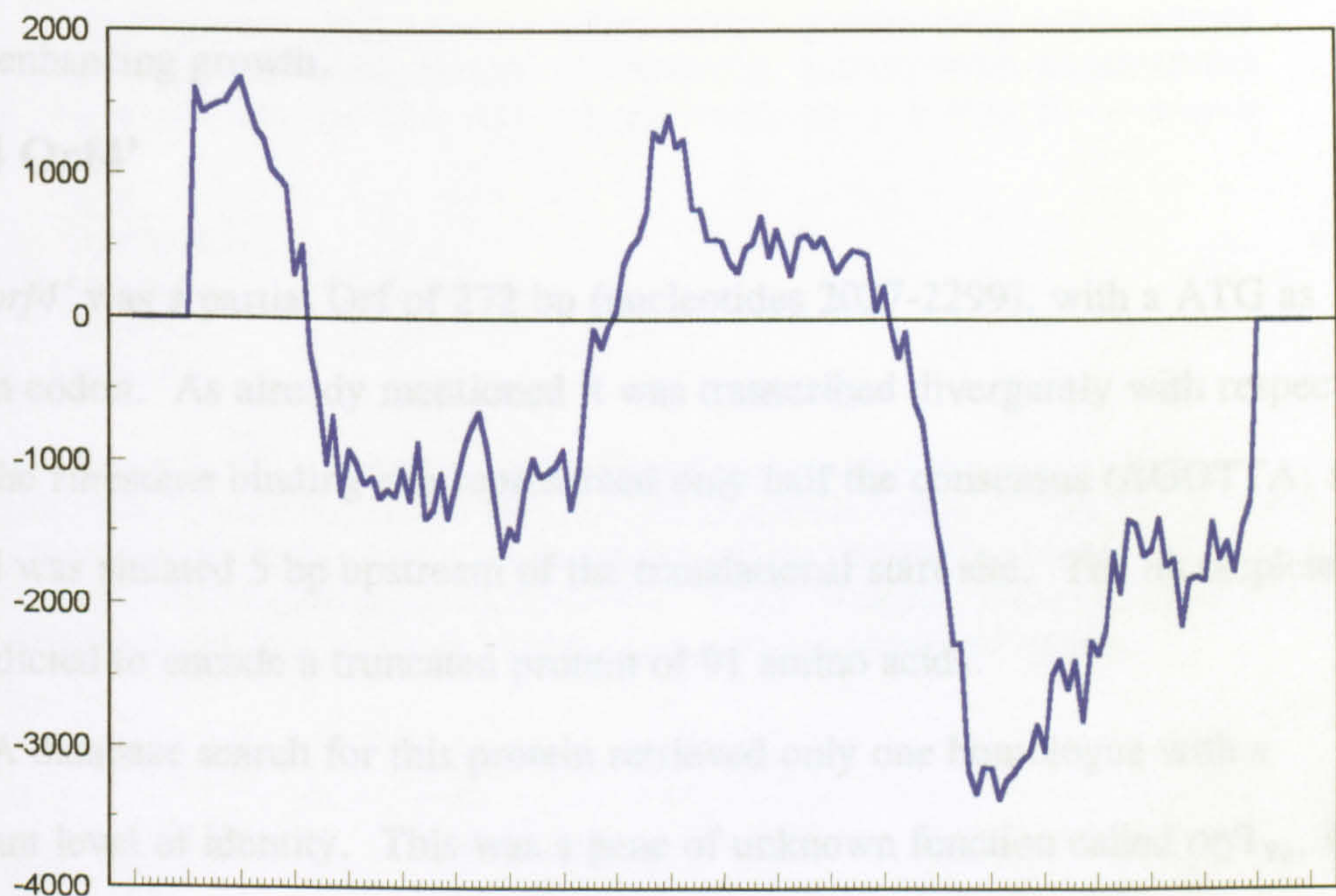
FIGURE 4.8

Figure 4.8 The predicted hydropathy profile of the protein product of *orf3* drawn using TMpred.

The window for this program was set at 17 residues and scores in excess of 500 are considered to be significant in representing a membrane spanning domain. The prediction made from these data was that there were three possible transmembrane domains at residues 6-22, 60-81 and 83-100.

Both Pcp_{Y_e} and Pcp_{Hi} are outer membrane lipoproteins with the characteristic leader sequences and lipoprotein signal cleavage sites. Pcp_{Y_e} from *Yersinia enterocolitica* was thought to be a virulence factor involved with the uptake of iron chelators such as Desferrioxamine B which promote growth in iron starved environments (Baumler and Hantke, 1992). In high copy this gene did promote growth under-iron starvation conditions. However this was not due to an active transport system but was caused by a copy number effect whereby high amounts of Pcp caused perturbation of the outer membrane increasing its permeability to Desferrioxamine B, and thereby enhancing growth.

4.6.2.4 Orf4'

orf4' was a partial Orf of 272 bp (nucleotides 2027-2299), with a ATG as its initiation codon. As already mentioned it was transcribed divergently with respect to *orf3*. The ribosome binding site represented only half the consensus (AGGTTA; figure 4.5) and was situated 5 bp upstream of the translational start site. The incomplete *orf4'* was predicted to encode a truncated protein of 91 amino acids.

A database search for this protein retrieved only one homologue with a significant level of identity. This was a gene of unknown function called *orf1_{Y_e}*, it had been submitted to the database along with *pcp_{Y_e}* of *Yersinia enterocolitica* (Baumler and Hantke, 1992).

Orf1_{Y_e} shared 67% identity with Orf4' over the 91 amino acids encoded on pNRT324 (figure 4.10), the *orf1_{Y_e}* gene actually encoded a protein of 271 amino acids. The function of Orf1_{Y_e} was, as already mentioned, undetermined. The hydropathy profile (this study) of this protein predicted that it had two transmembrane α -helices from residues 74-94 and 171-193 (figure 4.11) of which Orf4' had the first between residues 57-81 (data not shown).

The N-terminus of the Orf1_{Y_e} protein appeared to have a hydrophobic region and the C-terminus appeared to be hydrophilic. The cellular localisation prediction was that

FIGURE 4.9

	1					50
Pcp _{Hi}	MKKT	NMALAL	LVA	FSVTGCA	NTDIFSGDVY	SASQAKEARS ITYGTIVSVR
Pcp _{Ye}	MIKPLI	AVAIA	IAAV	TLTGCA	NNNTLSGDVF	SASQAKQVQT VTYGTLLSVR
Orf3	MMKYFL	VIT	LASIT	LVGCA	NTSTLSGDVY	SASDAKQVQT VTYGTIISTR
Cons	-M-K-	-----	LAA-TLTGCA	NT-TLSGDVY	SASQAKQVQT	VTYGTI-SVR
	51					100
Pcp _{Hi}	PVKIQAD	NQGV	VVGT	LG	GGGAL	GGIAGSTIGG ...GRGQAIA AVVGAIGGAI
Pcp _{Ye}	PVTIQGG	DDN	NVMGA	IGGAV	LGGFLGNTVG	GGTGRSLATA AGAVAGGMAG
Orf3	PVQIQAGE	EN	NIIGAL	GGAV	LGGFLGNTVG	GGTGRSLATA AGAVAGGVAG
Cons	PV-IQAG	--N	NV-GA-GGAV	LGGFLGNTVG	GGTGRSLATA	AGAVAGG-AG
	101					150
Pcp _{Hi}	AGSKIEEK	MS	QVNGA	ELVIK	KDDGQEI	VVV QKADSSSFVAG RRVRI
Pcp _{Ye}	QGVQGA	MNRT	DGVQLE	VRKD	DGTTILV	VQK QGPTRFSVGQ RVMLASSGST
Orf3	QSVEGA	INRV	QGVQLE	IRKD	DGNTIMV	VQK QGTTKIFYAGQ RIAMVSDGRS
Cons	QGV-GA-NR-		QGVQLE-RKD	DG-TI-VVQK	QG-T-F-VGQ	R----S-G-S
	151					
Pcp _{Hi}	SLN	SVL				
Pcp _{Ye}	VTV	SPR.				
Orf3	VTV	SPR.				
Cons	VTV	SPR-				

Figure 4.9 A lineup of *orf3* and the lipoprotein homologues retrieved from the database. The lineup of Orf3 (*S. marcescens*, this study), Pcp_{Hi} (*Haemophilus influenzae*, Deich *et al.*, 1988; Accession number M18877) and Pcp_{Ye} (*Yersinia enterocolitica*, Baumler and Hantke., 1992; Accession number X60448) were compiled using PILEUP of the UWGCG package. The consensus is written below (Cons) indicating the position of at least 2 identical or conserved amino acid residues.

FIGURE 4.10

	1					50
Orf1 _{Ye}	MGNK	VIAMKS	GRFIGV	MSGT	SLDGIDV	VLA AIDERMVAQQ ASYCHPMPLQ
Orf4'	MKS	GRYIGV	MSGT	SLDGIDV	VLA AIDEYTVAQQ ASYCHPIPQS
Cons	-----	MKS	GR-IGV	MSGT	SLDGIDV	VLA AIDE--VAQQ ASYCHP-P--
	51					100
Orf1 _{Ye}	LKKDIL	GMCQ	GQSTTL	SAVG	KLESQ	LGILF AEAVLALLAK AGLTAQDITA
Orf4'	IRLAIL	SMCQ	GQPVTL	SALG	HLDTS	SVFCL TEAVLALLKQ SGISASDI..
Cons	----IL-MCQ	GQ--TL	SA-G	-L-----	-EAVLALL--	-G--A-DI--
	101					
Orf1 _{Ye}	IGCHG	QTVWH....				
Orf4'					
Cons	-----					

Figure 4.10 A lineup of Orf4' and Orf1_{Ye} from *Y. enterocolitica*. The lineup of Orf4' (*S. marcescens*, this study), and Orf1_{Ye} (*Yersinia enterocolitica*, Baumler and Hantke., 1992. Accession number X60448) was compiled using PILEUP of the UWGCG package. The consensus is written below (Cons) showing identical residues.

FIGURE 4.11

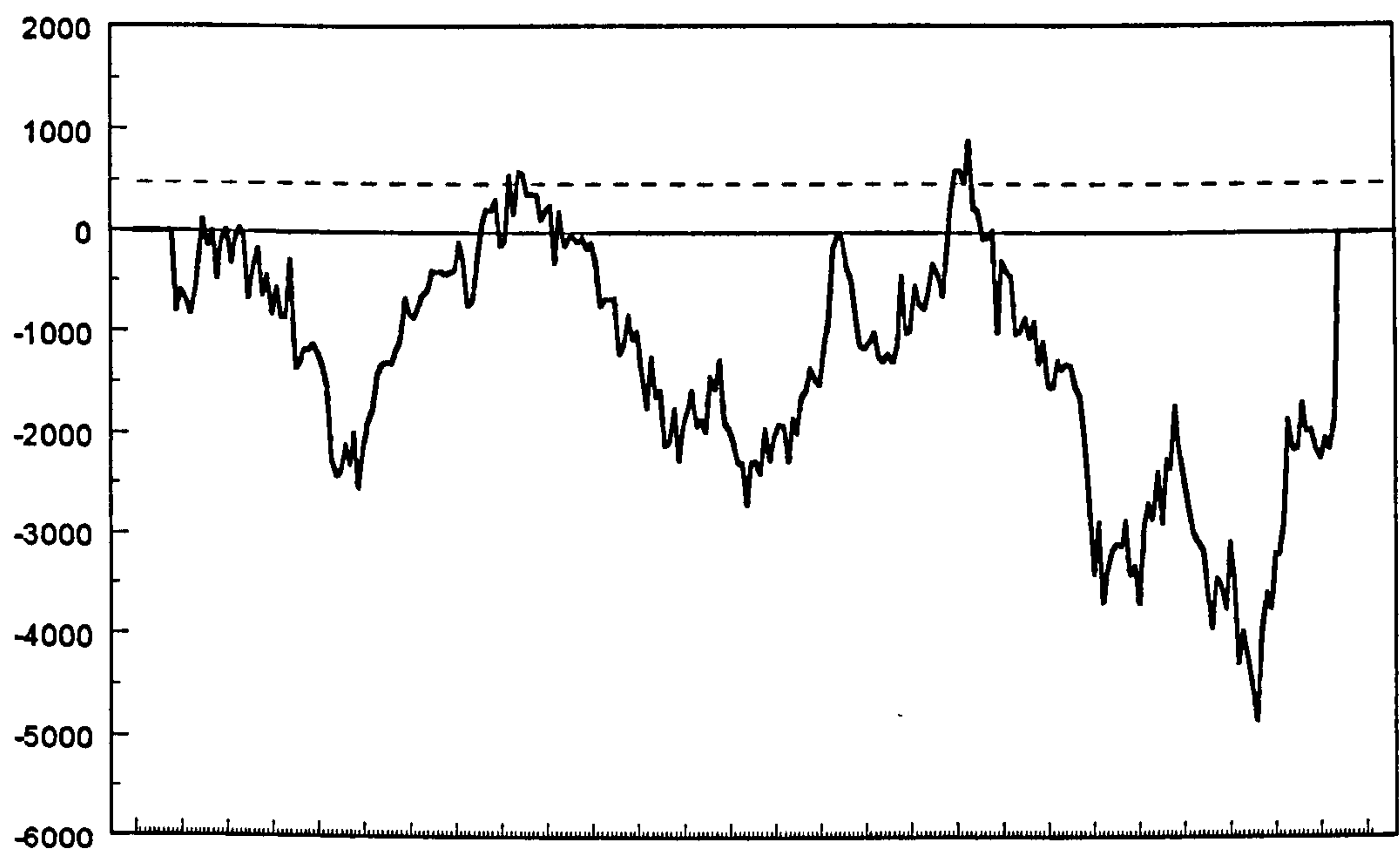


Figure 4.11 The predicted hydropathy profile of Orf1_{Ye} (using the sequence of Baumler and Hantke, [1992]; Accession number X60448) drawn using TMpred. The window for this program was set at 17 residues and scores in excess of 500 are considered to be significant in representing a membrane spanning domain. The prediction made from these data was that there were two possible transmembrane domains at residues 74-94 and 171-193

this was an inner membrane protein. Data base searches found no other protein with significant homology to either Orf1_{ye} or Orf4’.

4.7 CONCLUSIONS AND FINAL DISCUSSION

The insert of pNRT324 was sequenced, revealing two partial and two complete Orfs, three of which were found to share a high degree of homology with genes in the database. The plasmid pNRT324 contained only the 3’ end ‘orf1 which could not be expressed on pNRT324 and so was concluded to play no role in the complementation of the Rap phenotype.

The complete gene, *orf2*, was predicted to encode a protein which was homologous, with an identity of 71 %, to SlyA (Salmolysin), a protein discovered in *Salmonella typhimurium* (Libby *et al.*, 1994). SlyA was previously concluded to be a novel pore forming cytolysin able to lyse a variety of mammalian cells. The level of amino acid identity between Orf2 and SlyA declined towards the C-terminus which may indicate that the N-terminus is the functional domain, or the structural basis of some differences in the function of these highly conserved proteins. It is important to note that no haemolytic activity was observed for *E.coli* cells carrying any plasmid containing *orf2*. Database searches failed to find any homologues from previously reported haemolysins or any other recorded protein.

There are several points regarding SlyA that were at issue with the preliminary findings of this study. The features of the two well characterised families of pore forming haemolysins of Gram negative bacteria, typified by the α -haemolysin from *E.coli* (HlyA) and the haemolysin encoded by *shlA* of *S. marcescens* (Welch, 1991), are summarised in table 4.2.

Table 4.2 highlights the differences between previously characterised haemolysins and SlyA which led to it being classed as a novel cytolysin. The most striking difference between SlyA and the other types of haemolysin was the length of the protein. SlyA was only 9% of the size of ShlA, being more comparable in size to the

TABLE 4.2

Taken from Braun *et al* (1993).

RTX-family α-haemolysin- <i>E.coli</i> (HlyA)	ShlA- <i>S.marcescens</i>
Operon 4 genes	Operon 2 genes
Haemolysin HlyA (1024aa)	Haemolysin ShlA (1578aa)
Activation by HlyC (170aa)	Activation by ShlB (539 aa)
Secretion requires HlyB (707aa) and HlyD (478aa)	Secretion requires ShlB .
Secretion also requires TolC	Secretion does not require TolC
No N-terminal signal sequence	N-terminal signal sequence .
Activation in cytoplasm	Activation in periplasm
Dependent on Ca ²⁺	Independent of Ca ²⁺
RTX motif	No RTX motif
Secretion motif in C-terminus	Secretion motif in N-terminus
Pore-forming domain in N-terminus	Pore-forming domain in C-terminus
Membrane recognition in C-terminus	Membrane recognition in N-terminus
Forms pores in erythrocyte membranes	Forms pores in erythrocyte membranes

Table 4.7 Comparison of the RTX and ShlA family of Gram negative pore forming haemolysins
Taken from Braun *et al.*, (1993) showing the two well characterised classes of pore-forming haemolysins of Gram negative bacteria, typified by α-haemolysin of uropathogenic *E.coli* (HlyA) and the *Serratia* haemolysin ShlA.(Key; aa- amino acids; RTX- repeats in toxins).

thiol-activated haemolysins of Gram positive bacteria such as Listeriolysin from *Listeria monocytogenes* (529 amino acids; Mengaud *et al.*, 1989) and α -toxin of *Staphylococcus aureus* (293 amino acids; Gray and Kehoe, 1984).

SlyA's lack of an N-terminal signal sequence was not uncharacteristic of haemolysins: *hlyA* of uropathogenic *E.coli* forms part of an operon which includes *hlyB* and *hlyD*, the products of which are responsible for the secretion of HlyA in a one step *sec*-independent pathway (reviewed by Braun and Focareta, 1991). It is not possible to tell if *slyA* was part of a similar operon, because of the lack of surrounding sequence reported. However, from the sequence of pNRT324 it was obvious that *orf 2* was not part of an operon and there were no obvious, complete, genes nearby which could perform analogous functions to *hlyB* and *hlyD* for its secretion.

The work reported here forced a further critical analysis of the conclusions of Libby *et al.*, on several other points: Firstly, it is now evident that homologues of SlyA are not restricted to *Salmonella*, *Shigella* and enteroinvasive *E.coli* and are found in *S.marcescens*. Secondly, Libby *et al.*, (1994) reported a significant stretch of hydrophobic residues at the C-terminus of SlyA. However this was not evident from the hydropathy profiles produced for SlyA and Orf2 in this study. The protein prediction made from my analysis classified SlyA and Orf2 as cytoplasmic proteins, consistent with the absence of an N-terminal signal sequence.

It has been reported (reviewed by Welch, 1991) that *E.coli* haemolysins in sublethal doses can have profound effects on their host cells, for example enhancing the rate of phagocytosis of bacteria by neutrophils and inducing the release of inflammatory mediators (Scheffer *et al.*, 1985). It is difficult to see how this could explain the distinct Rap phenotype in *Serratia*.

Following the completion of the lab based research for this study, an article by Ludwig *et al.*, (1995) was published. This study set out to critically evaluate the findings of Libby *et al.*, (1994). They reported that various *E.coli* strains when transformed with a *slyA* encoding vector were indeed haemolytic. However, using anti-

SlyA antibodies, they found that this haemolytic phenotype, although induced by *slyA*, was in fact caused by a previously uncharacterised cryptic *E.coli* haemolysin and not as had been suggested by Libby *et al.*, by ‘Salmolysin’. In addition, *slyA* null mutants of *S.typhimurium* were found to be affected in the production of many proteins and so, they concluded that SlyA is not a haemolysin but a global regulatory protein. Therefore the findings of Ludwig *et al.*, (1995) are consistent with the preliminary findings of this study, relating to Rap.

The complete gene *orf3* encoded a protein with a characteristic lipoprotein signal sequence and cleavage site. The prediction made from the amino acid sequence immediately downstream of the cleavage site was that this protein would be localised to the outer membrane (Yamaguchi *et al.*, 1988; see figure 6.9 for details). These data were taken to strongly suggest that the protein product of Orf3 was a lipoprotein.

Database searches revealed Orf3 was homologous to Pcp_{Ye} and Pcp_{Hi}, lipoproteins from *Yersinia enterocolitica* and *Haemophilus influenzae* respectively. The function of Pcp_{Ye} as a possible virulence factor was investigated (discussed in section 4.6.2.3; Baumler and Hantke, 1992) but its role was equivocal. The role of many lipoproteins in Gram negative bacteria (especially pathogens) is thought to be one of a sensor with respect to pathogen-host interactions, e.g. TraT which is involved with serum resistance and inhibition of phagocytosis by macrophage (reviewed by Sukupolvi and O’Connor, 1990).

Deletion mutants of Pcp_{Ye} produced by Baumler and Hantke, (1992) were neither affected in growth nor virulence and so the function of this lipoprotein in *Yersinia* remains unclear. However over-expression of Pcp_{Ye} caused perturbation of the outer membrane which is thought to be the reason why the subclone pNRT370 and pNRT345 appeared to be deleterious to the *E.coli* host, and in turn responsible for the difficulties encountered when subcloning pNRT300.

The results of this study and those of Baumler and Hantke, (1992) and Diech *et al.*, (1988) revealed that Pcp homologues are highly conserved in animal and human pathogens. This suggests a selective pressure for the retention of these genes.

To confirm that *orf3* did indeed encode a lipoprotein would require further investigation: such as the use of globomycin (Inukai *et al.*, 1978). Globomycin specifically inhibits the activity of signal peptidase II, preventing the processing of prelipoprotein. On treatment with globomycin, cells accumulate prelipoprotein which can be visualised on a SDS-PAGE gel in which, if Orf3 were a lipoprotein, only the higher molecular weight precursor would be seen.

Interestingly the gene product of *orf4*' was found to be homologous to a protein of unknown function (*orf1_{Y_e}*) located upstream of *Pcp_{Y_e}* (Baumler and Hantke, 1992). The gene arrangement of *orf1_{Y_e}* and *pcp_{Y_e}* from *Yersinia* was almost identical to their homologues discovered in this study, *orf3* and *orf4*'. The conservation of sequence and genetic organisation suggests some common ancestry for this 'cassette' or perhaps may be due to horizontal transfer between *S. marcescens* and *Yersinia*.

The sequence of pNRT324 did not produce an obvious candidate regulatory protein for the control of antibiotic and pigment production since there were no classical DNA binding motifs found in any of the gene products. Another possibility was that the DNA on pNRT324, and not an Orf, was titrating out a regulatory protein (perhaps a repressor) and suppressing the mutant phenotype. An example of this effect would be the *gal* operator region which, on a high copy plasmid, binds the *gal* repressor (Irani *et al.*, 1983). However the use of the low copy vector pSF6 should have prevented this.

To determine which of the genes found on pNRT324 was responsible for the Rap phenotype it was first necessary to identify which of these genes (or which DNA region) was responsible for the complementation of the *S. marcescens* Rap⁻ mutants. The identification of 'Rap' is discussed in the next chapter.

***IDENTIFICATION OF rap AND
ITS
HOMOLOGUES***

5.0 PREFACE

As described previously the Rap cosmid pNRT300 was isolated by its ability to complement the pleiotropic *Serratia* Rap mutants to Car⁺, Pig⁺. The complementing clone was subcloned to a 2.3kb *EcoRV*-*SalI* fragment and sequenced. On this fragment were two complete and two partial Orfs. The first partial Orf, denoted 'orf1, had no promoter, ribosome binding site or translation start codon and so was considered unlikely to have a role in the Rap phenotype. The gene product of *orf2* shared a high level of identity with SlyA (Salmolysin) from *Salmonella*; a virulence factor required for survival in macrophages. The third open reading frame *orf3* was similar to the lipoproteins *pcp_{Ye}* and *pcp_{Hi}* from *Yersinia* and *Haemophilus* respectively and *orf4*' was homologous to a gene (*orf1_{Ye}*) of unknown function also from *Yersinia*.

To identify which, if any, of these Orfs was responsible for the regulation of antibiotic and pigment (the *rap* gene), it was necessary to isolate the Orfs encoded on pNRT324. The sequence of the 2.3kb *EcoRV*-*SalI* fragment of pNRT324 was known (Chapter 4) and this facilitated the cloning of various fragments from this construct.

5.1 SUBCLONING THE pNRT324 PLASMID

Plasmid pNRT324 was cut with *AseI* which generated two fragments: a 1.3 kb band, containing *orf3* and *orf4*', and a larger 4.6 kb band, containing 1.2 kb of insert DNA, covering *orf2* and 'orf1 and 3.4 kb of vector DNA (figure 5.1). The 4.6 kb band was self-ligated (forming pNRT312) and the 1.3 kb band was ligated into pACYC184 (Chang and Cohen, 1978) cut with *AseI* (forming pNRT313). After confirming the success of the cloning in *E.coli* strain DH1, the plasmids were used to transform NT5. Of the two subclones only pNRT312 complemented NT5 for antibiotic and pigment production.

The most likely candidate for the *rap* gene was considered to be *orf2* because 'orf1 could not be expressed on pNRT312. To investigate whether *orf2* was responsible

FIGURE 5.1

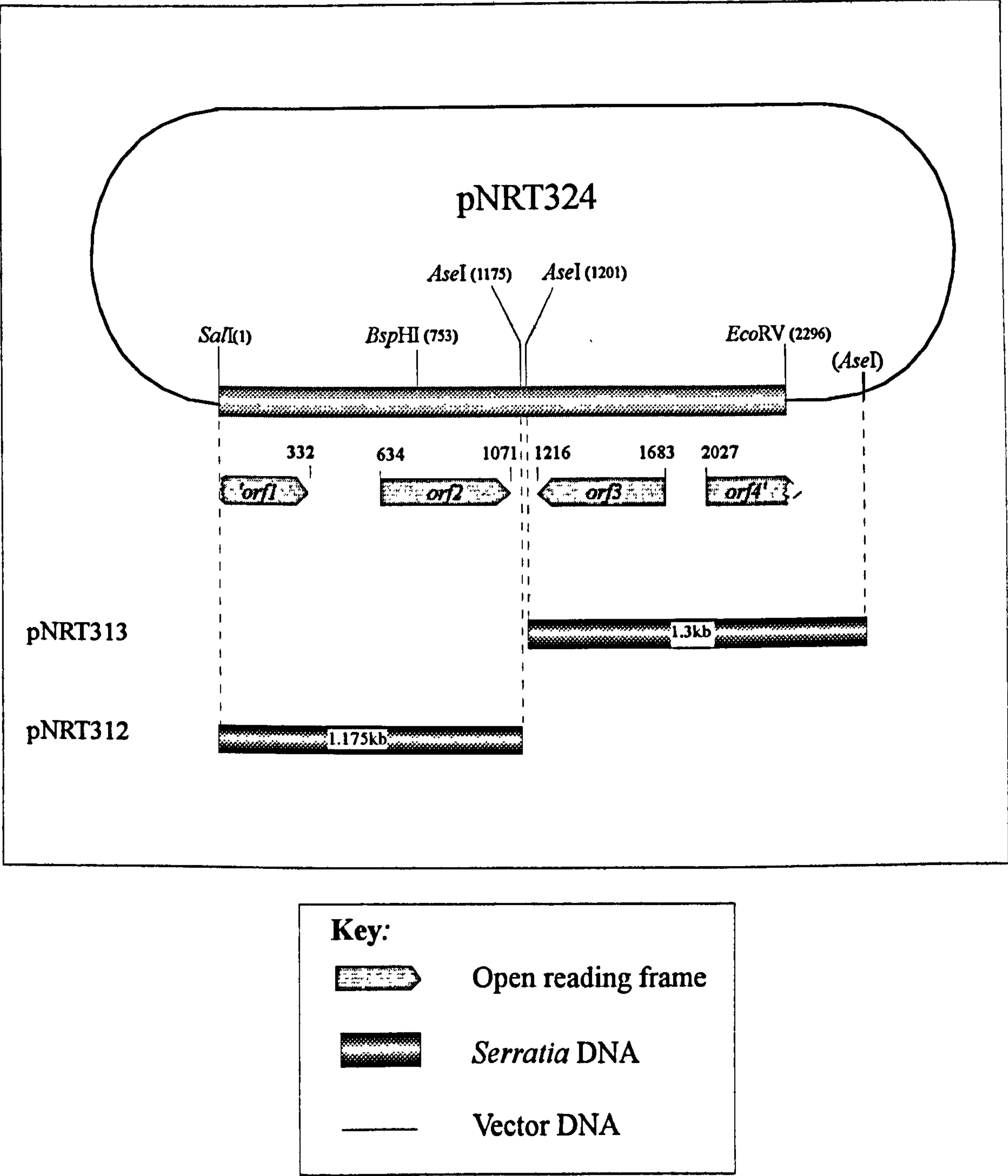


Figure 5.1 A schematic restriction map of pNRT324. Important restriction sites used to subclone and identify the *rap* gene are shown. The positions of the Orfs and restriction enzyme cleavage sites are marked (in base pairs), with the first base of the left hand end of the insert as position 1. The subclones generated from pNRT324 are drawn below, with the names they were designated down the side. The size of the insert DNA contained in each subclone is given in kb.

for this complementation a mutation was engineered into the open reading frame as detailed in the following section.

5.2 THE INTRODUCTION OF A STOP CODON INTO THE *S.marcescens slyA* HOMOLOGUE: *orf2*

The restriction map of pNRT324 was used to identify unique restriction sites which cut in *orf2* (figure 5.1). Plasmid pNRT312 was digested with *Bsp*HI, then end repaired using Klenow and religated. The result of these manipulations was a shift in the reading frame leading to the introduction of a stop codon and therefore premature termination of translation of *orf2* (as shown in figure 5.2). This mutant allele was denoted *orf2'* and the plasmid carrying it, pNRT312B. The predicted truncated protein encoded by *orf2'* was 42 amino acids in length (compared to the wild type 146 amino acids) as shown in figure 5.3. To confirm the success of this manipulation the sequence of the mutated *orf2'* was determined (data not shown) and then NT5 was transformed with pNRT312B. Transformants were selected on NBA supplemented with chloramphenicol. Plasmid pNRT312B failed to complement for pigment and carbapenem production in NT5.

These results show that the gene responsible for the Rap phenotype was *orf2*. The genes encoded by pNRT324 were renamed now that the identity of *rap* was known: '*orf1* was renamed as '*orfY*, *orf2* was renamed as *rap*, *orf3* was named *pcp_{Sm}* and *orf4*' as *orfX*'. Identifying *rap* also meant that the EMS mutants (NT2,5-9), generated at the very beginning of this study (section 3.0), could be characterised, which would further confirm the identity of the *rap* gene and may also identify important functional domains within its protein product - Rap.

5.3 STRUCTURE AND FUNCTION MAPPING OF Rap

5.3.1 INTRODUCTION

Rap mutants were originally isolated during the study of 5R-carbapen-2-em-3-carboxylic acid production by *S.marcescens* (section 3.0). Of the colonies surviving

FIGURE 5.2

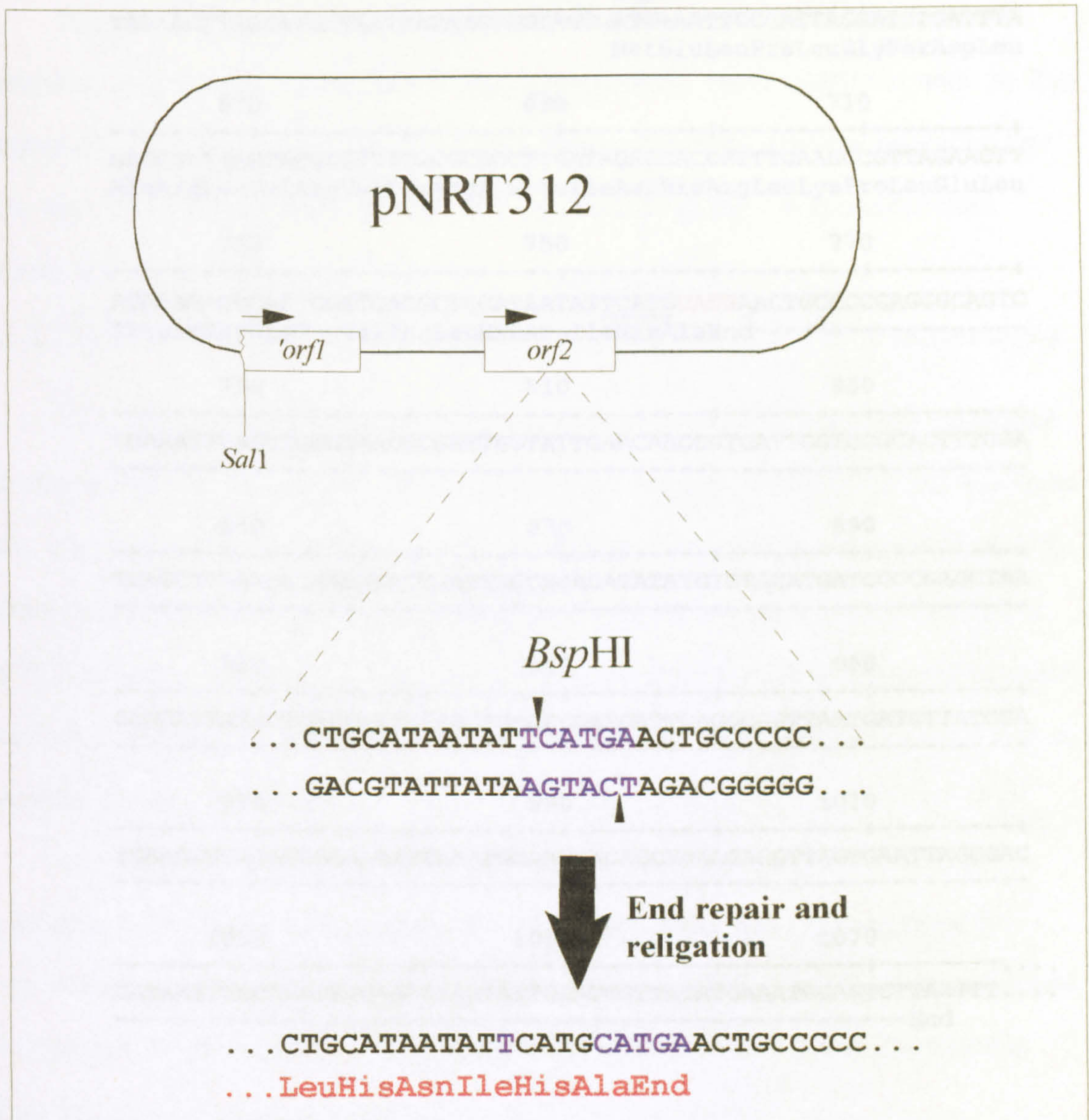


Figure 5.2 The introduction of a translational stop codon into *orf2*.

Plasmid pNRT312 was cut with *BspHI*, end repairing and religating. The enzyme cleavage site is marked by arrow heads and the sequence introduced as a result of end repair is shown in bold with the *BspHI* restriction site shown in blue. The amino acid translation is written below the sequence

FIGURE 5.3

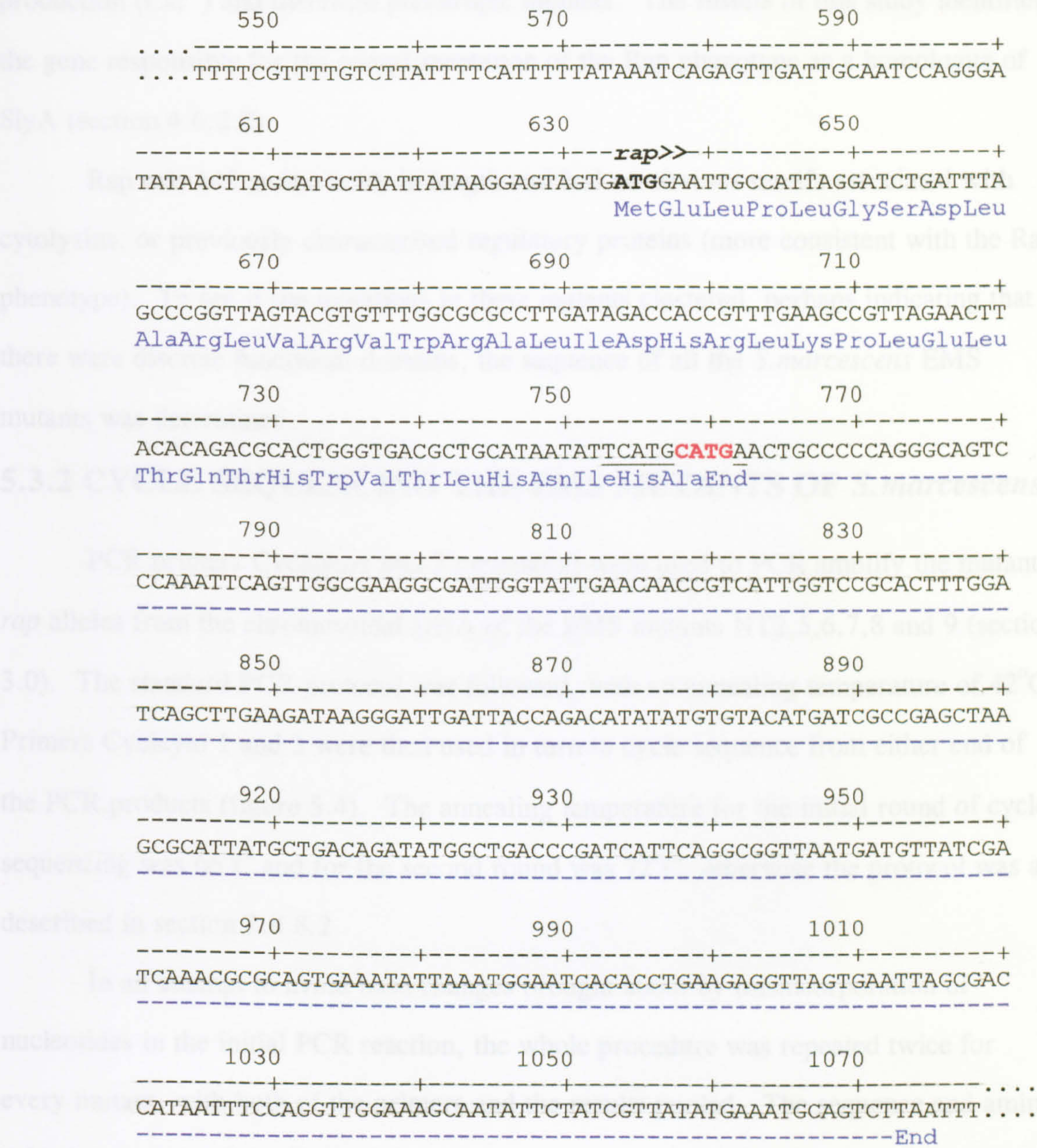


Figure 5.3 The introduction of a stop codon into *rap*.
By cutting, endfilling and religating of pNRT312, 4bp of sequence (shown in red) were introduced into *orf2*. The resultant frame shift introduced a TGA stop codon into the open reading frame. The predicted truncated protein was 42 amino acids long (shown in blue) in comparison with the wildtype protein of 145 amino acids (represented by the blue dashed line ending in the termination codon TAA). Nucleotides underlined indicate the original *Bsp*HI site.

EMS mutagenesis some were phenotypically white (Pig γ) and defective for antibiotic production (Car γ) and therefore pleiotropic mutants. The results of this study identified the gene responsible for the complementation of the Rap phenotype as a homologue of SlyA (section 4.6.2.2).

Rap was 145 amino acids in length and had no obvious motifs associated with cytolysins, or previously characterised regulatory proteins (more consistent with the Rap phenotype). To see if the mutations in these mutants clustered, perhaps indicating that there were discrete functional domains, the sequence of all the *S.marcescens* EMS mutants was determined.

5.3.2 CYCLE SEQUENCING THE EMS MUTANTS OF *S.marcescens*

PCR primers Cyclcyto1 and 3 (appendix) were used to PCR amplify the mutant *rap* alleles from the chromosomal DNA of the EMS mutants NT2,5,6,7,8 and 9 (section 3.0). The standard PCR protocol was followed, with an annealing temperature of 42°C. Primers Cyclcyto 1 and 3 were then used in turn to cycle sequence from either end of the PCR products (figure 5.4). The annealing temperature for the initial round of cycle sequencing was 65°C and for the second round was 72°C, otherwise the protocol was as described in section 2.8.8.2.

In an attempt to avoid base changes brought about by misincorporation of nucleotides in the initial PCR reaction, the whole procedure was repeated twice for every mutant, with both of the primers and the results pooled. The sequence and amino acid changes for all the EMS *rap* mutants are recorded in table 5.1 and illustrated in figure 5.5

5.3.3 ANALYSIS OF THE RESULTS FROM THE CYCLE SEQUENCING OF THE EMS *rap* MUTANTS

As can be seen from figure 5.5 all of the mutations were mapped to positions within the Orf, with the exception of NT6 which was positioned within the putative ribosome binding site. All of the base changes were characteristic of those generated by

FIGURE 5.4

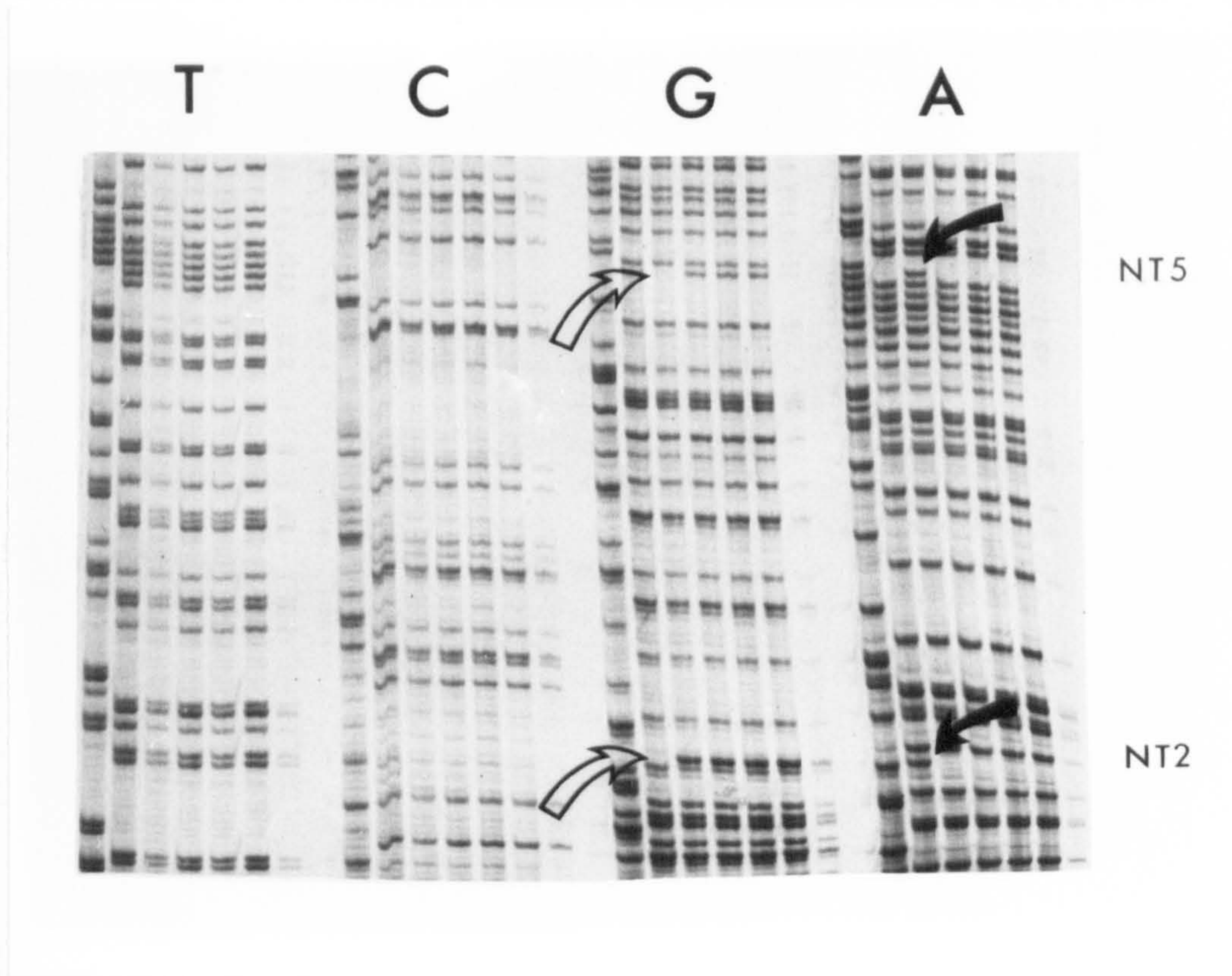


Figure 5.4 The sequence of the EMS induced *rap* gene mutants NT5 and NT2.

Empty arrows indicate the original position of the guanine nucleotide and the solid arrows point to the position of the resultant base change after EMS mutagenesis. A - adenine; T - thymine; C - cytosine; G - guanine.

FIGURE 5.5

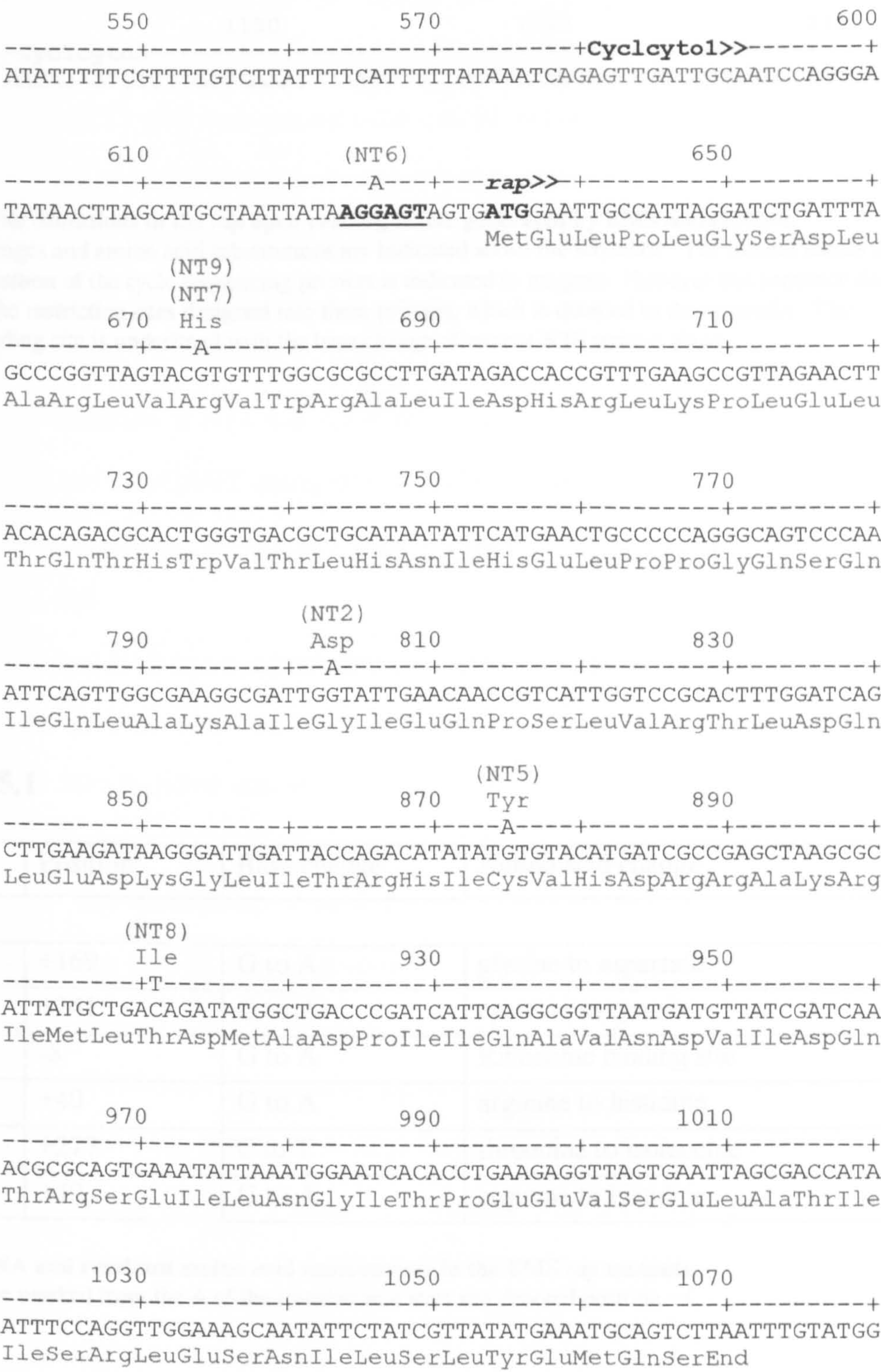


FIGURE 5.5

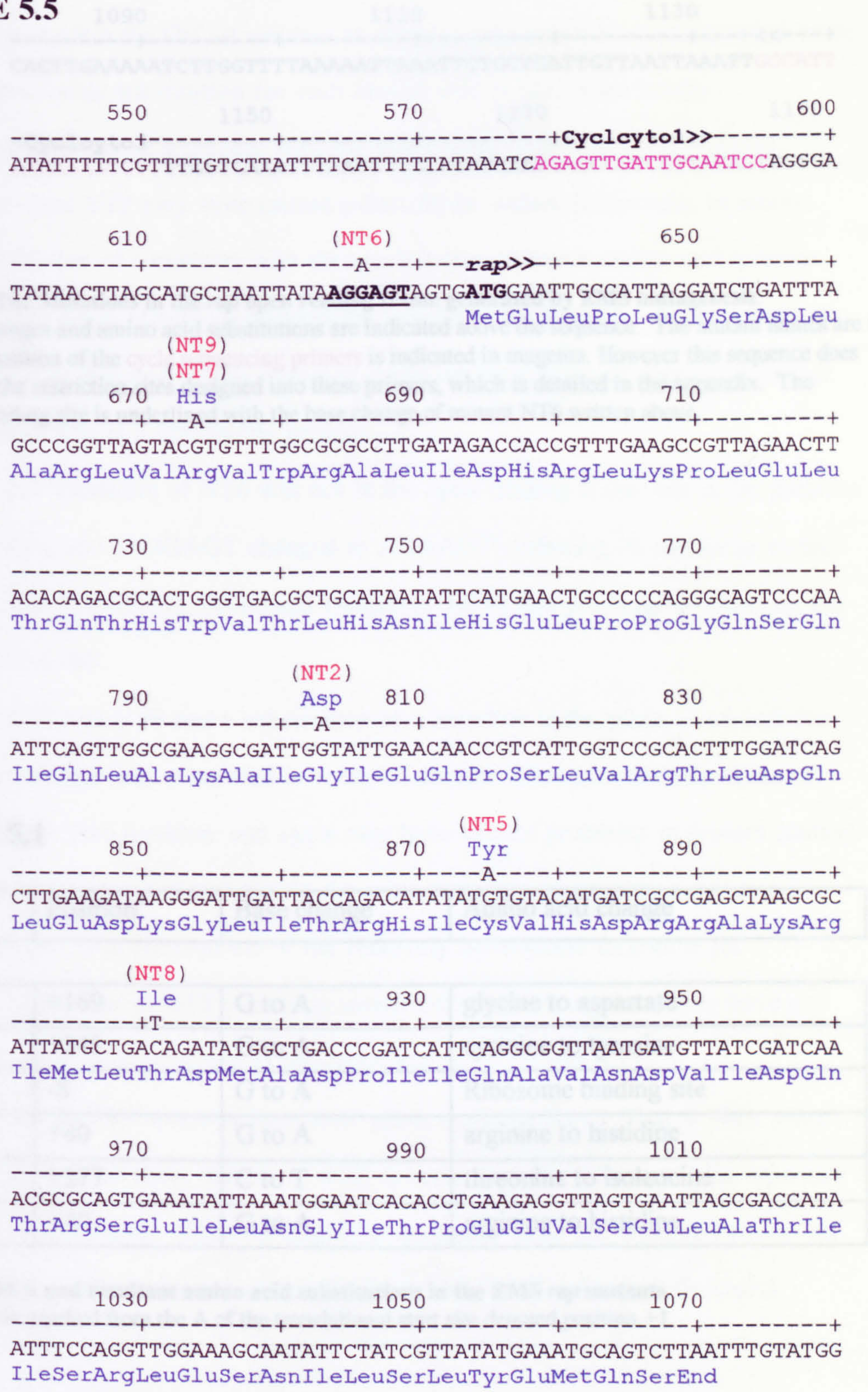
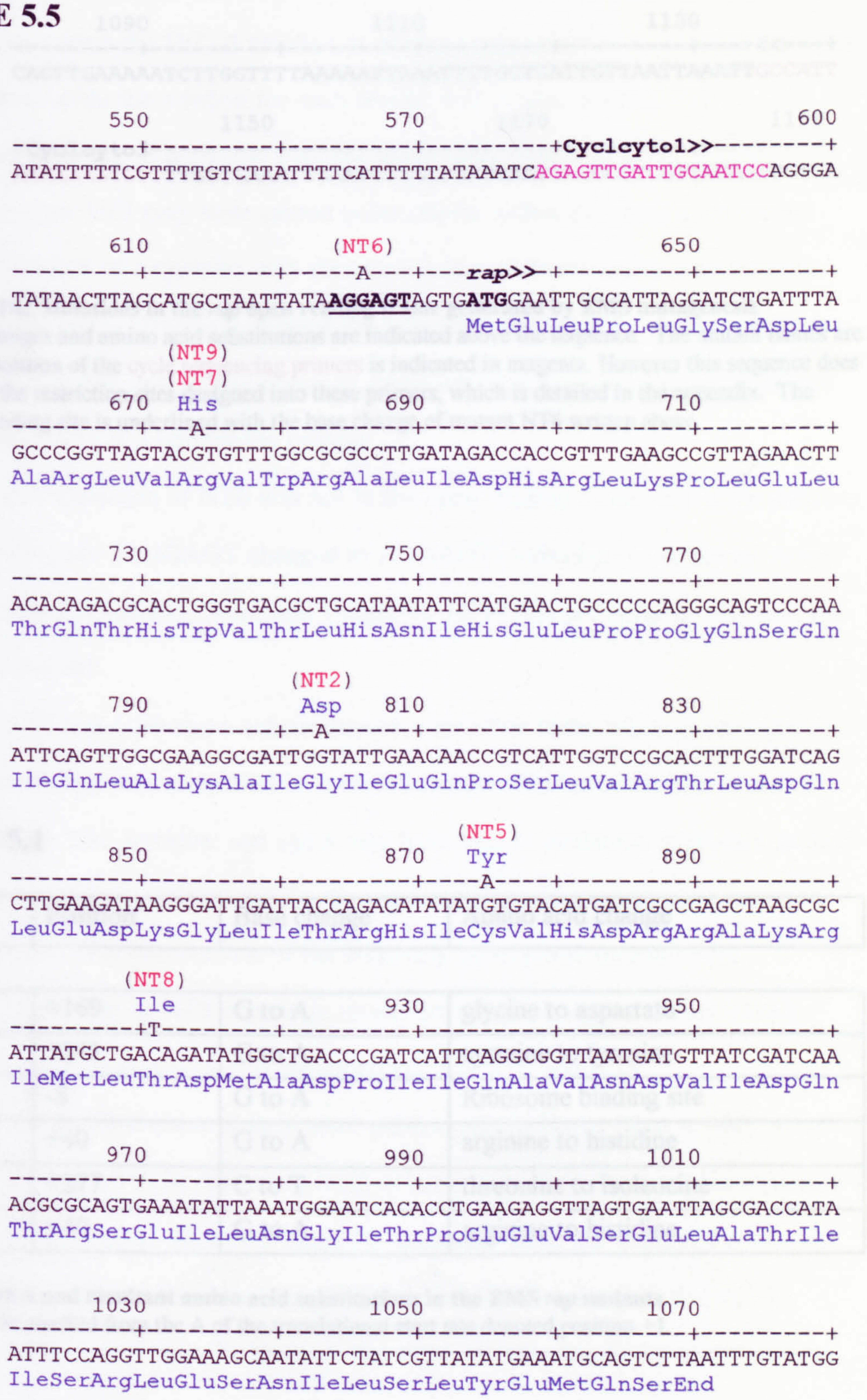


FIGURE 5.5



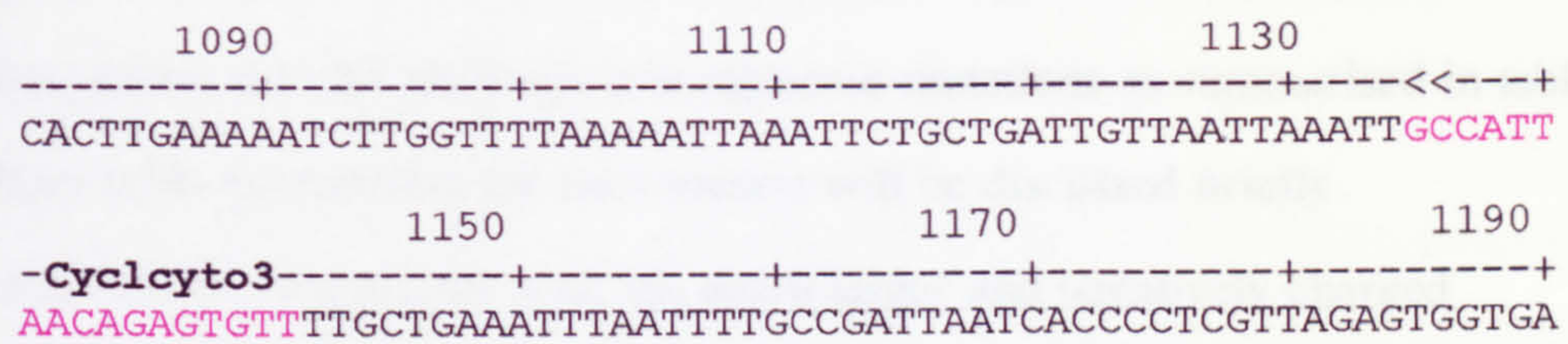


Figure 5.5 The Mutations in the *rap* open reading frame generated by EMS mutagenesis. The base changes and amino acid substitutions are indicated above the sequence. The mutant names are in blue. The position of the cycle sequencing primers is indicated in magenta. However this sequence does not include the restriction sites designed into these primers, which is detailed in the appendix. The ribosome binding site is underlined with the base change of mutant NT6 written above.

TABLE 5.1

Mutant	Position ¹	Base change	Amino acid change
NT2	+169	G to A	glycine to aspartate
NT5	+241	G to A	cysteine to tyrosine
NT6	-8	G to A	Ribosome binding site
NT7	+40	G to A	arginine to histidine
NT8	+277	C to T	threonine to isoleucine
NT9	+40	G to A	arginine to histidine

Table 5.1 DNA and resultant amino acid substitutions in the EMS *rap* mutants.
¹ Positions are marked from the A of the translational start site denoted position +1.

EMS mutagenesis; either guanine - adenine or cytosine - thymine base transitions. The base transitions within the Orf all resulted in missense mutations as summarised in table 5.1. The amino acids substitution for each mutant will be discussed briefly.

The substitution of a glycine with the much larger and negatively charged aspartate in mutant NT2 may have caused polar effects within the protein. In mutant NT5 the substitution of a cysteine with the much larger aromatic amino acid tyrosine may have caused steric effects on perhaps the folding of the protein. There is only one cysteine residue in Rap and so this mutation could not have affected disulphide bridge formation unless Rap is a multimeric protein.

The base transition of NT6 was not in the open reading frame but in the putative ribosome binding site (AGGAGT changed to AGAAGT) reducing its similarity to the *E.coli* consensus (AGGAGG; Shine and Dalgarno, 1974) and presumably affecting the translation of the Orf.

Both NT7 and NT9 had a substitution of a histidine in the place of an arginine residue. This change was conserved in terms of charge. However the side chain of arginine is longer than histidine and again may have caused problems in protein folding. These two mutants were isolated during the same mutagenesis experiment and so are probably siblings. The substitution of the relatively hydrophilic threonine for hydrophobic isoleucine in NT8 was not a conservative substitution and may have a similar effect on protein structure as the mutations for NT6 and NT9.

All the mutants sequenced here were phenotypically Rap⁻. That is, they were Pig⁻ and Car⁻ and so all these mutations must in some way have disrupted the structure or function of the protein. However since the mutations were not clustered within the gene little can be inferred from individual substitutions until a detailed structural analysis has been carried out. To increase the value of these results far more mutants would have to be isolated and sequenced.

5.4 INACTIVATION OF *rap* AND *pcp_{Sm}* BY ALLELIC EXCHANGE

5.4.1 INTRODUCTION

Marker exchange was used to construct definitive and selectable chromosomal mutations within both *rap* and *pcp_{Sm}*. It was considered important to define the role of *pcp_{Sm}*, if any, in the Rap phenotype because of its proximity to *rap* and the fact that the function of this lipoprotein and its homologues is unknown.

Marker exchange was carried out using the suicide vector pKNG101 (Kaniga *et al.*, 1991). The marker exchange involved two homologous recombination events; In the first stage, the integration of the vector into the chromosome was forced by the plasmid vector only being able to replicate in a host supplying the *pir* gene product *in trans*. These recombinants were recovered by the selection of the streptomycin antibiotic resistance marker encoded by the plasmid. Secondly the resolution of the plasmid and the wild type allele was achieved by selecting on NBA containing 10% sucrose, and because the mutant *rap* allele was disrupted by the kanamycin resistance gene, antibiotic was also used as a selection.

The vector contains *sacB*, a gene encoding levansucrase. This enzyme catalyses the hydrolysis of sucrose as well as the synthesis of levans, which are lethal to many Gram negative bacteria in the presence of high concentrations of sucrose (Gay *et al.*, 1985). This acted as the selection, forcing a secondary recombination event and only then allowing the bacteria to survive on high sucrose media.

The mutant alleles of *rap* and *pcp_{Sm}* to be exchanged were constructed and cloned into the marker exchange vector pKNG101 as described in the following sections.

5.4.2 CONSTRUCTION OF *rap* AND *pcp_{Sm}* MARKER EXCHANGE PLASMIDS

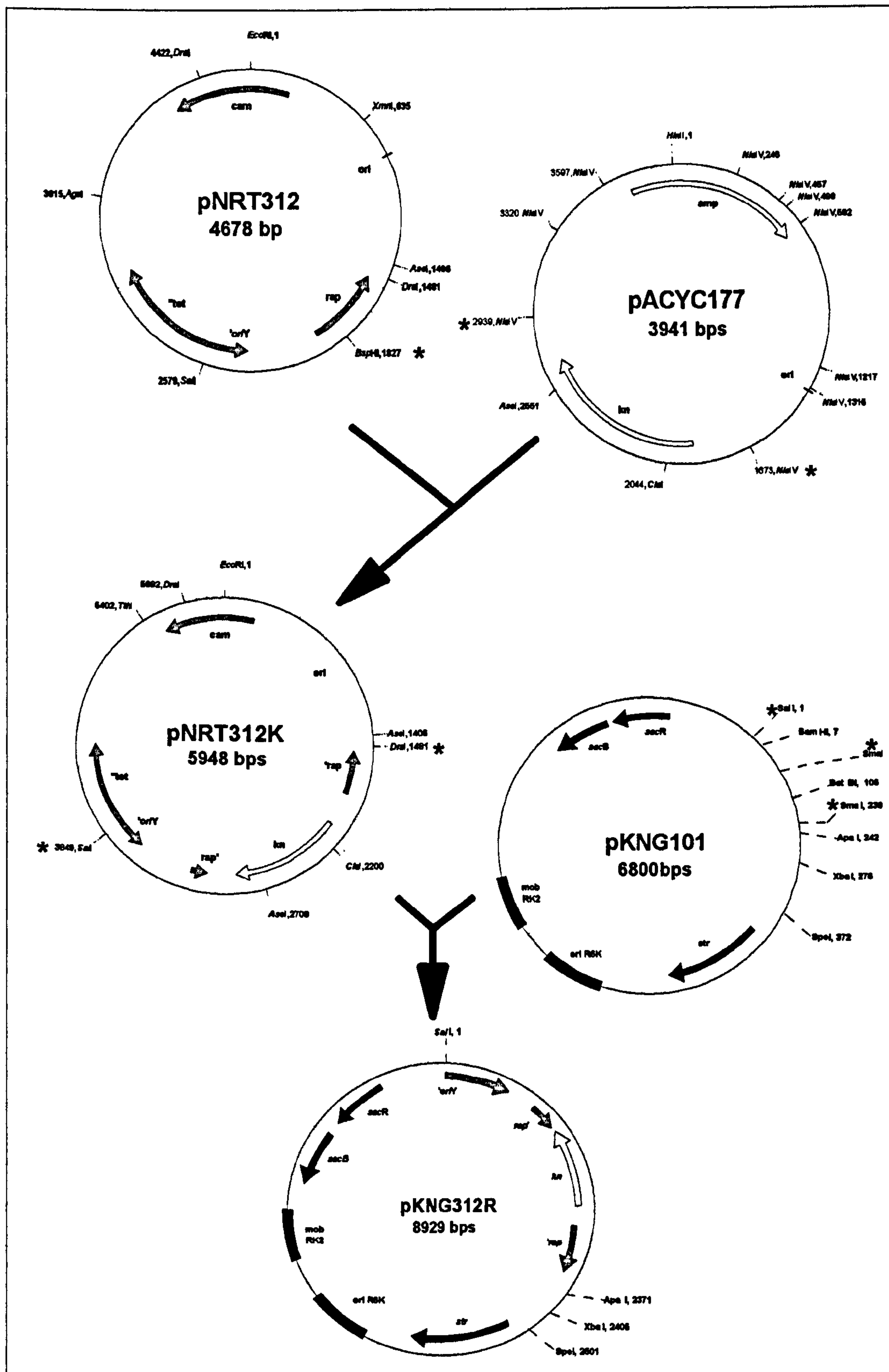
The construction of the plasmids for marker exchange was similar for both of the genes and is illustrated in figure 5.6 and figure 5.7 for *rap* and *pcp_{Sm}* respectively. A kanamycin resistance cassette, cut out of plasmid pACYC177 (Chang and Cohen, 1978;

Legend to figure 5.6

The strategy for the construction of the *rap* marker exchange plasmid pKNG312R.

The kanamycin cassette from pACYC177 was excised on a 1266bp blunt ended restriction fragment using *Nla*IV. This cassette was then ligated into the unique *Bsp*HI site of pNRT312. *E.coli* strain DH1 was transformed with pNRT312K and selected on NBA supplemented with chloramphenicol and kanamycin. The 2368bp *Dra*I - *Sa*II fragment was cut from pNRT312R and ligated into the compatible *Sma*I - *Sa*II sites of pKNG101 to give pKNG312R. This ligation was used to transform *E.coli* strain CC118 (λ *pir*) and transformants were selected on NBA supplemented with streptomycin and kanamycin. Genes are shaded corresponding to their parental plasmid. The published sequence of pKNG101 has a unique *Sma*I site. However there is a second *Sma*I site between the published *Sma*I site and the *Sa*II site (S.McGowan, pers.comm). This necessitated the use of *Sa*II before *Sma*I because *Sa*II cannot cut efficiently close to the end of a DNA fragment. (Plasmid resistance gene abbreviations: *kn*- kanamycin resistance; *str*- streptomycin resistance; *tet*- tetracyclin resistance; *amp*- ampicillin resistance; *cam*- chloramphenicol resistance.

FIGURE 5.6

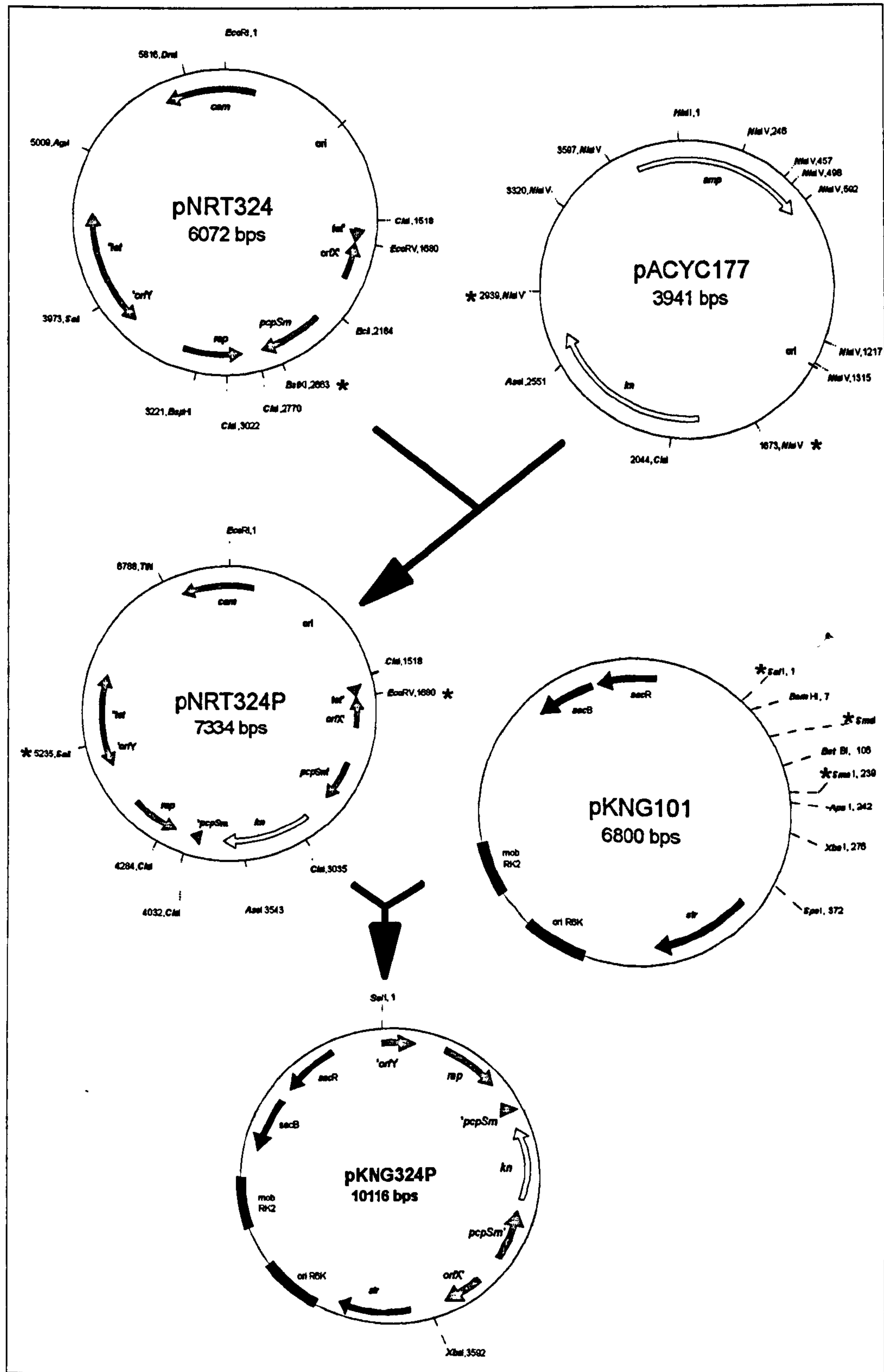


Legend to figure 5.7

The strategy for the construction of the *pcp_{Sm}* marker exchange plasmid pKNG324P.

The 1266 bp kanamycin cassette was excised as from pACYC177 using *Nla*IV and ligated into the unique *Bst*XI site (end-repaired using T4 polymerase) of pNRT324 and transformants selected on NBA supplemented with chloramphenicol and kanamycin. The 3555bp *Eco*RV - *Sal*I fragment containing the kanamycin cassette was cut from pNRT324P and ligated into pKNG101 cut with *Sal*I- *Sma*I. This ligation was electroporated into *E.coli* strain CC118 (λ *pir*) and transformants were selected on NBA supplemented with streptomycin and kanamycin. Genes are shaded corresponding to their parental plasmid. (Plasmid resistance gene abbreviations: *kn*- kanamycin resistance; *str*- streptomycin resistance; *tet*- tetracyclin resistance; *amp*- ampicillin resistance; *cam*- chloramphenicol resistance.

FIGURE 5.7



appendix), was cloned into both *rap* and *pcp_{sm}* thereby disrupting the open reading frame and allowing direct selection of the mutant allele. The insertion of the kanamycin cassette left a predicted truncated protein of 42 amino acids for Rap, encoded on pKNG312R (wild type Rap is 145 amino acids in length), and 124 amino acids for Pcp_{sm}, encoded on pKNG324P (wild type Pcp_{sm} is 155 amino acids in length).

E. coli strain CC118 (λ pir) was transformed with pKNG312R or pKNG324P and used for marker exchange as previously described (section 2.10). The kanamycin antibiotic resistance allowed the direct selection of the desired recombination event, leaving a chromosomally-encoded mutant kanamycin resistant allele (*kan^R*) after marker exchange. The outcome of the recombination events and the antibiotic and sucrose selection used during the integration and resolution of pKNG312R (*rap::kan^R*) marker exchange plasmid is described in figure 5.8 and 5.9 respectively

The events leading to the marker exchange of *pcp_{sm}* were the same as those for *rap* and so are not shown. The phenotypes used to select the Pcp_{sm} marker exchanged mutant were kanamycin resistance, streptomycin sensitive and sucrose resistant.

The marker exchange of *rap* after the selection on high sucrose and kanamycin, led to the recovery of mutant strains which were Pig⁻ and when tested on the *E. coli* strain ESS carbapenem bioassay were Car⁻: the Rap phenotype. The recovered *pcp_{sm}* marker exchange mutants were unaffected for pigment or antibiotic production. To confirm the marker exchange had been successful Southern blots were carried out for both *rap* and *pcp_{sm}* (discussed in sections 5.4.3 and 5.4.4 respectively).

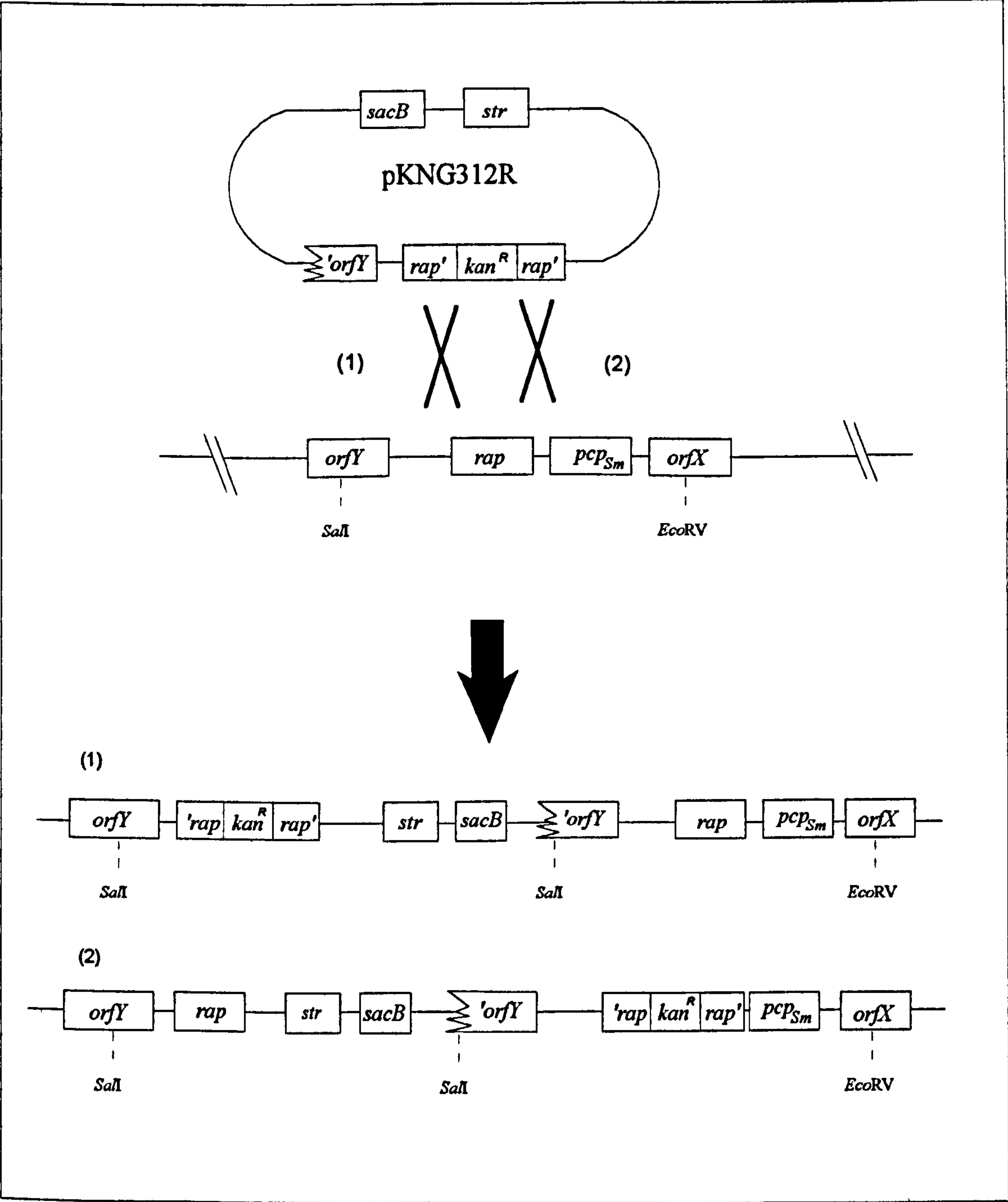
5.4.3 SOUTHERN BLOT TO CONFIRM MARKER EXCHANGE OF *rap*

Chromosomal DNA was prepared from a number of the mutants recovered after marker exchange. The DNA was cut with *EcoRV* and *SalI* restriction enzymes, which do not cut in the kanamycin cassette (figure 5.9), and prepared for a Southern blot as previously described. If the marker exchange was successful a band shift equal to the size of the kanamycin cassette would be observed (1266bp). The chromosomal DNA

Legend to Figure 5.8

Integration of pKNG312R into the chromosome of *Serratia marcescens* ATCC39006.
Plasmid pKNG312R encoded the disrupted *rap::kan^R* and the partial '*orfY*'. Integration of the plasmid took place by homologous recombination. There were two possible outcomes dictated by the position of the cross-over with respect to *rap::kan^R* allele, both resulting in colonies resistant to streptomycin and kanamycin, sensitive to high concentrations of sucrose and diploid with respect to *rap*. (Gene abbreviations- *kan^R* -kanamycin resistance, *str* -streptomycin resistance)

FIGURE 5.8

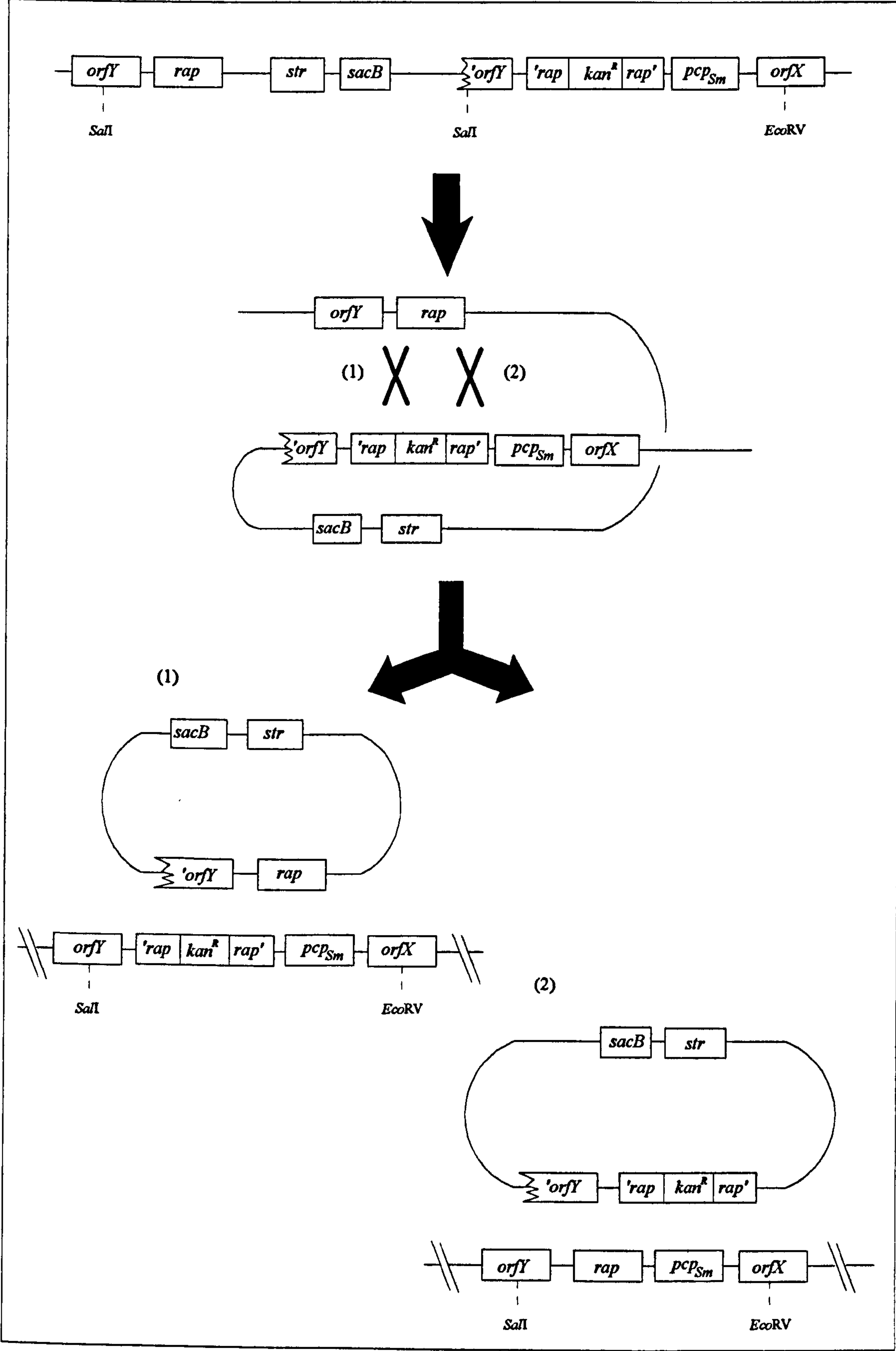


Legend to Figure 5.9

Resolution of the integrated plasmid pKNG312R.

The integrated plasmid pKNG312R in the chromosome of *S.marcescens* was flanked by regions of homology. Recombination between these two regions resulted in "looping out" of the plasmid. The position of the cross-over event relative to *rap::kan^R* dictated the genotype of the resultant strain. A cross-over at position 1 would loop out a plasmid encoding a wild type *rap* gene, leaving the disrupted *rap::kan^R* allele in the chromosome. This strain was kanamycin resistant, streptomycin sensitive, resistant to high concentrations of sucrose and Rap⁻. Alternatively following a cross-over at position 2 the strain would be kanamycin and streptomycin sensitive resistant to high sucrose and Rap⁺. The desired recombination event at position 1, was selected for by supplementing the media with 10% sucrose and kanamycin. The excised plasmid was lost because the *S.marcescens* host does not have the λ *pir* gene required for its maintenance. (Plasmid resistance gene abbreviations- *kan^R* -kanamycin resistance, *str* -streptomycin resistance)

FIGURE 5.9



(figure 5.10a) was probed with a 474bp PCR product (made using primers Cyto1 and Cyto2, and labelled with digoxigenin-11-dUTP - DIG; see appendix for primers) containing the *rap* Orf.

The Southern blot confirmed that the marker exchange was successful (figure 5.10b). The wild type control had a hybridising band of ~2300 bp and the marker exchange mutants had hybridising bands of ~3600 bp equal to the control band size plus the 1266 bp kanamycin cassette.

5.4.4 SOUTHERN BLOT TO CONFIRM MARKER EXCHANGE OF *pcp_{Sm}*

Chromosomal DNA was prepared from five kanamycin resistant strains recovered after marker exchange, all of which were pigmented and produced carbapenem. The DNA was cut with *EcoRV*-and *SalI* (figure 5.11a) and probed in a Southern blot exactly as for section 5.4.3. The probe was a 548 bp PCR probe, containing the *pcp_{Sm}* Orf, made using primers PCPS1 and PCPS2 (appendix) and labelled with DIG (section 2.7.2).

The Southern blot (figure 5.11b) shows the band shift equal to the insertion of the kanamycin cassette confirming the marker exchange was successful. The *pcp_{Sm}* marker exchange mutants were indistinguishable from the wild type for all phenotypes tested including:- production of antibiotic, pigment and the exoenzymes pectate lyase and cellulase. The growth rate was also identical to that of the wild type *S.marcescens* strain (data not shown).

5.5 DISCUSSION

The *rap* gene was successfully isolated on a 1.2 kb *EcoRV* - *SalI* fragment containing 'orf1 and orf2. The complete Orf2 was considered the most likely candidate for Rap and so a translational stop codon was engineered into the open reading frame (forming Orf2'). When orf2' was introduced into NT5 (plasmid pNRT312B) it failed to complement antibiotic and pigment production and as a result identified *orf2'* as *rap*.




FIGURE 5.10a

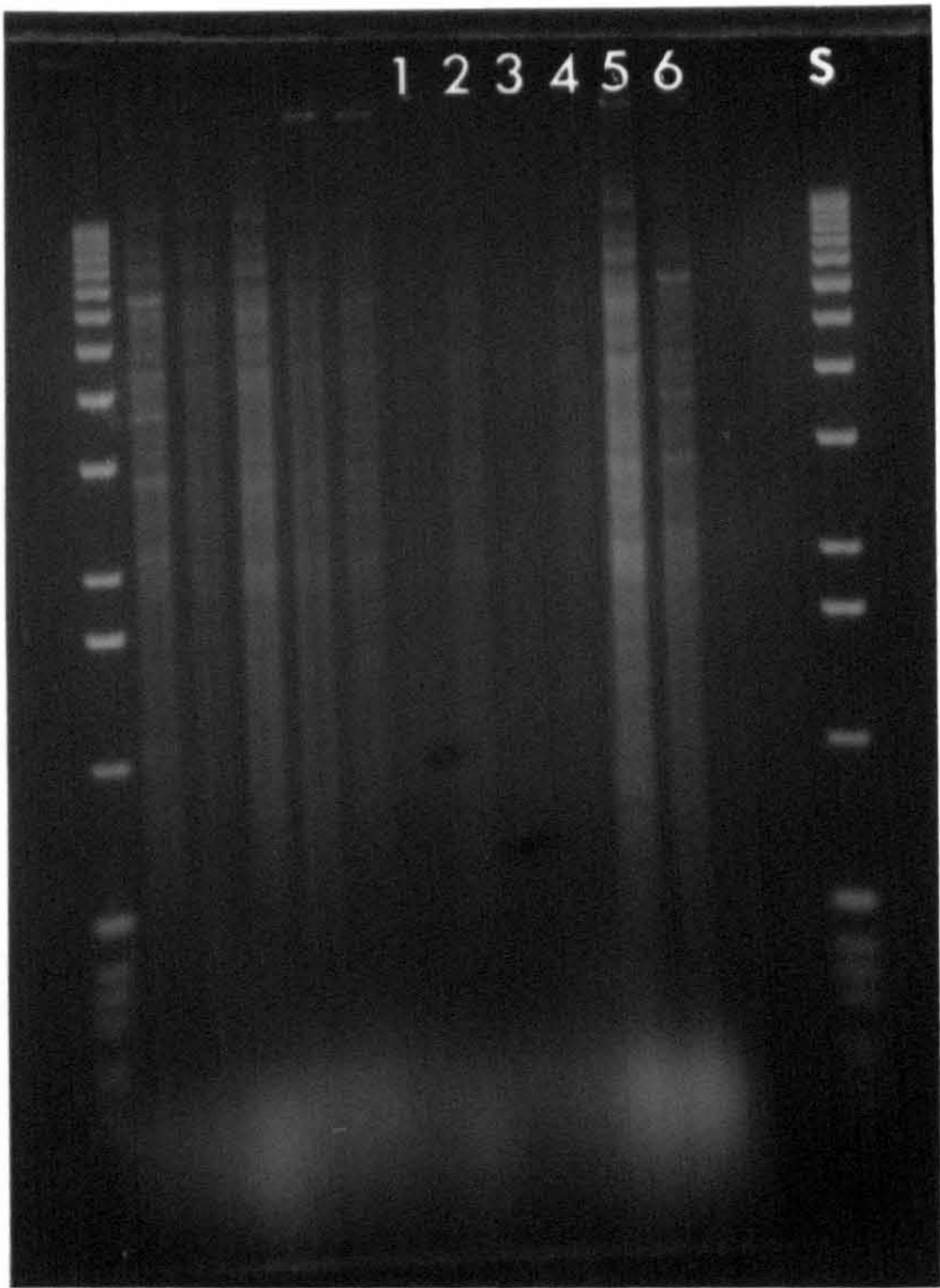


Figure 5.10a Restriction enzyme digests of *rap* marker exchange mutants.
Chromosomal DNA was cut with restriction enzymes *EcoRV* - *SalI*

Key to figures 5.10a and 5.10b				
Chromosomal DNA from:				
Lane:				
1	Marker exchange mutant	NTM1		
2	"	"	"	NTM2
3	"	"	"	NTM3
4	"	"	"	NTM4
5	"	"	"	NTM5
6	Wild type <i>S.marcescens</i> ATCC39006			
S	1 kb ladder			

FIGURE 5.10b

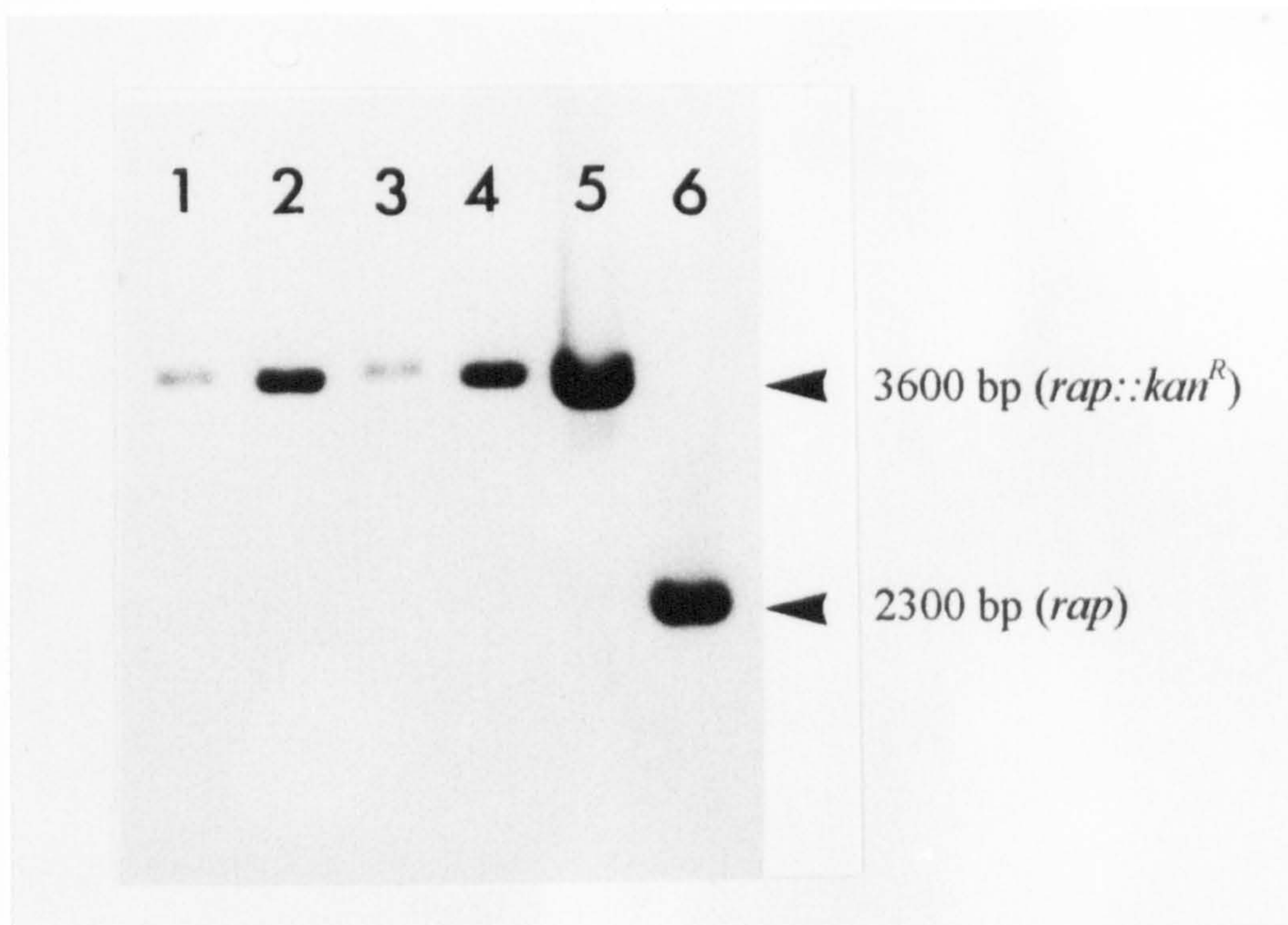


Figure 5.10b Southern blot of *rap* marker exchanged strains of *S.marcescens* ATCC39006.

Chromosomal DNA was probed with a 474 bp *rap* DIG probe made by PCR. The chromosomal DNA cut with *EcoRV*-*SalI* and gave a hybridising band of ~2300 bps for the wild type in lane 6. All the 'marker exchanged' strains (lanes 1-5) exhibited a hybridising band of ~3600 bps equal to the size of the *rap* containing, *EcoRV*-*SalI*, band plus the kanamycin cassette cloned into the *rap* Orf.

FIGURE 5.11a

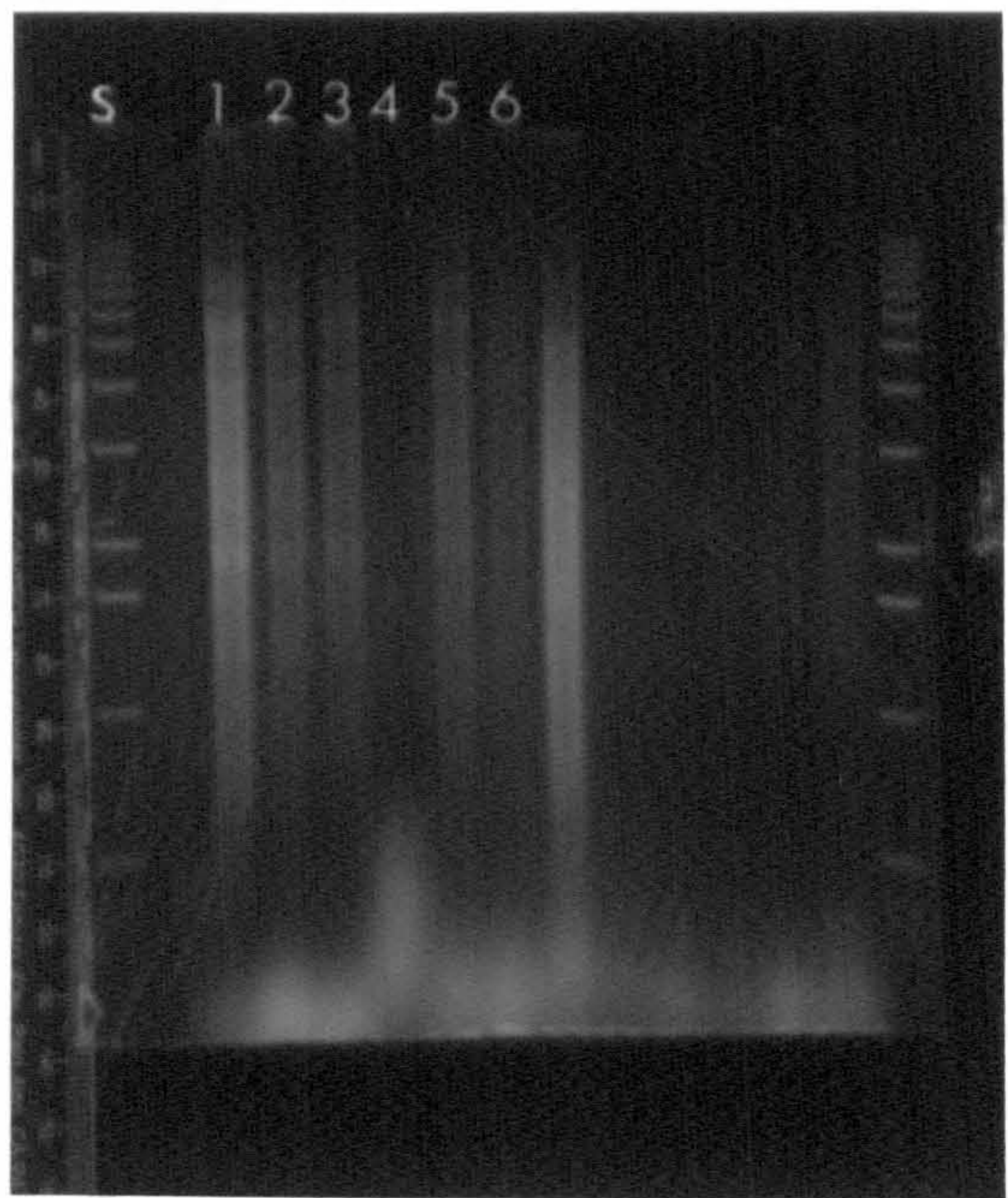


Figure 5.11a Restriction enzyme digests of *pcp_{sm}* marker exchange mutants. Chromosomal DNA was cut with restriction enzymes *EcoRV* - *SalI*

Key to figures 5.11a and 5.11b					
Chromosomal DNA					
Lane: S	1 kb ladder				
1	Wild type <i>S.marcescens</i> ATCC39006				
2	Marker exchange mutant	NTP1			
3	"	"	"	"	NTP2
4	"	"	"	"	NTP3
5	"	"	"	"	NTP4
6	"	"	"	"	NTP5

FIGURE 5.11b

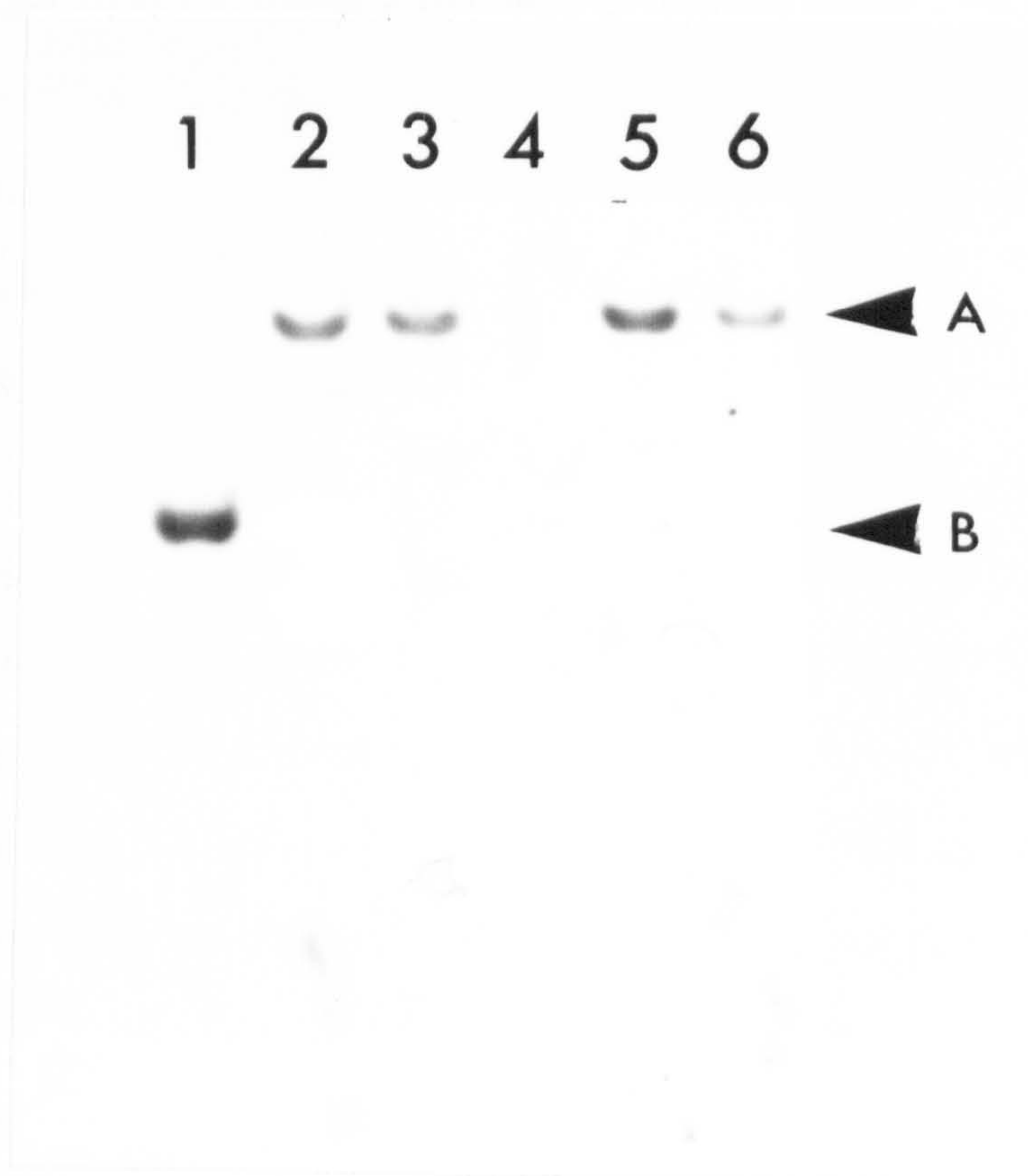


Figure 5.11b Southern blot to confirm the marker exchange of *S.marcescens* pcp_{Sm} .

Chromosomal DNA was probed with a 548 bp pcp_{Sm} DIG probe made by PCR. The chromosomal DNA was cut with *EcoRV*-*Sall* and gave a hybridising band of ~2300 bps (**B**) for the wild type in lane 1. All the 'marker exchanged' strains (lanes 2-6) exhibited a hybridising band of ~3600 bps (**A**) equal to the size of the pcp_{Sm} containing, *EcoRV*-*Sall*, band plus the kanamycin cassette cloned into the pcp_{Sm} Orf.

The identification of the *rap* gene allowed primers to be designed in order to sequence all of the EMS Rap⁻ mutants; NT2,5-9. The nucleotide changes for all these mutants were, except for NT6, in the open reading frame and were evenly distributed. The fact that the mutations did not cluster may suggest that in a protein of only 145 amino acids the majority of the residues are crucial for the overall function of the protein and that there are unlikely to be any discrete domains. It was interesting to note however, that all of the substitutions were in residues totally conserved between Rap and SlyA implying that these amino acids are of some importance. As more mutants are sequenced these data will perhaps aid in the identification of functionally important residues in the Rap protein.

EMS mutant NT6 had a mutation in the *rap* putative ribosome binding site. Mutations of this type have been reported previously: ribosome binding to the mRNA of gene 0.3 of phage T7 is destabilised by the change of one base from GAGG to GAAG (Lewin, 1987). It is evident that the base transition in NT6 appears to have had a profound effect on translation of this protein, based on the Rap⁻ phenotype.

The marker exchange of the *rap* gene was successful and the mutants recovered after marker exchange exhibited the characteristic, Pig⁻ and Car⁻, Rap phenotype. The production of a chromosomally defined mutation was considered to be the definitive experiment, proving that *orf2/rap* was solely responsible for the Rap phenotype observed originally in the EMS mutants.

The marker exchange of *pcp_{Sm}* was successful and demonstrated that under the conditions used in the laboratory this gene has no role in the regulation of antibiotic and pigment, or in fact in any phenotype tested (section 5.4.4). The mutation was not lethal and growth rate was unaffected. This data agreed with the results from the studies of the Pcp homologues from *Yersinia* and *Haemophilus* discussed in section 4.7.

5.6 IDENTIFICATION OF *rap* HOMOLOGUES BY SOUTHERN HYBRIDISATION

The isolation of *rap* from *S.marcescens* contradicted the findings of Libby *et al.*, (1994), in which *slyA* was only found in *Salmonella*, *Shigella* and enteroinvasive *E.coli*. The fact that *Serratia* had a homologue of SlyA left open the formal possibility that there were other homologues of *rap* which were also not detected by the study of Libby *et al.*

To determine the distribution of the homologues of *rap* a probe (made as in section 5.4.3 but labelled with α 32 P-dCTP) was used against chromosomal DNAs of various bacteria from our laboratories strain collection (figure 5.12).

Chromosomal DNAs were prepared from plant pathogens from the 'erwinias', including three subspecies of *Erwinia carotovora*: *carotovora* (*Ecc*), *atroseptica* (*Eca*) and *betavsculorum* (*Ecb*), as well as *Erwinia chrysanthemi* (*Ech*). The *Ecc* strains ATCC39048, SCRI124 and SCRI132 all make a carbapenem antibiotic (Bainton *et al* 1992a; M. Holden pers. comm.).

The *Serratia marcescens* strains tested included *S. marcescens* strain ATCC39006 which was used as a positive control for the Southern blot. The 'Sutton Bonington' *S. marcescens* strain (*Sbon*) which produces a small diffusible signalling molecule (G.Stewart pers. comm.) and a clinical isolate of *S. marcescens* strain S6 (*Sclin*) which was Pig⁻ and haemolytic, exhibiting a halo on sheep blood agar plates (Livermore, 1992). Other bacteria tested included *Escherichia coli* strain DH1, human and animal pathogens such as *Proteus mirabilis*, *Yersinia enterocolitica* and *Enterobacter agglomerans*. In addition *Salmonella typhimurium* DNA was represented in an attempt to detect *slyA*. Chromosomal DNA was digested with *Hind*III and prepared for a Southern blot as previously described (section 2.7).

The results of the Southern blot showed a *rap* hybridising band for all of the *Erwinia* species tested (figure 5.12). There were two main sizes of hybridising fragment observed; for the subspecies *carotovora* the majority of the hybridising bands were

FIGURE 5.12a

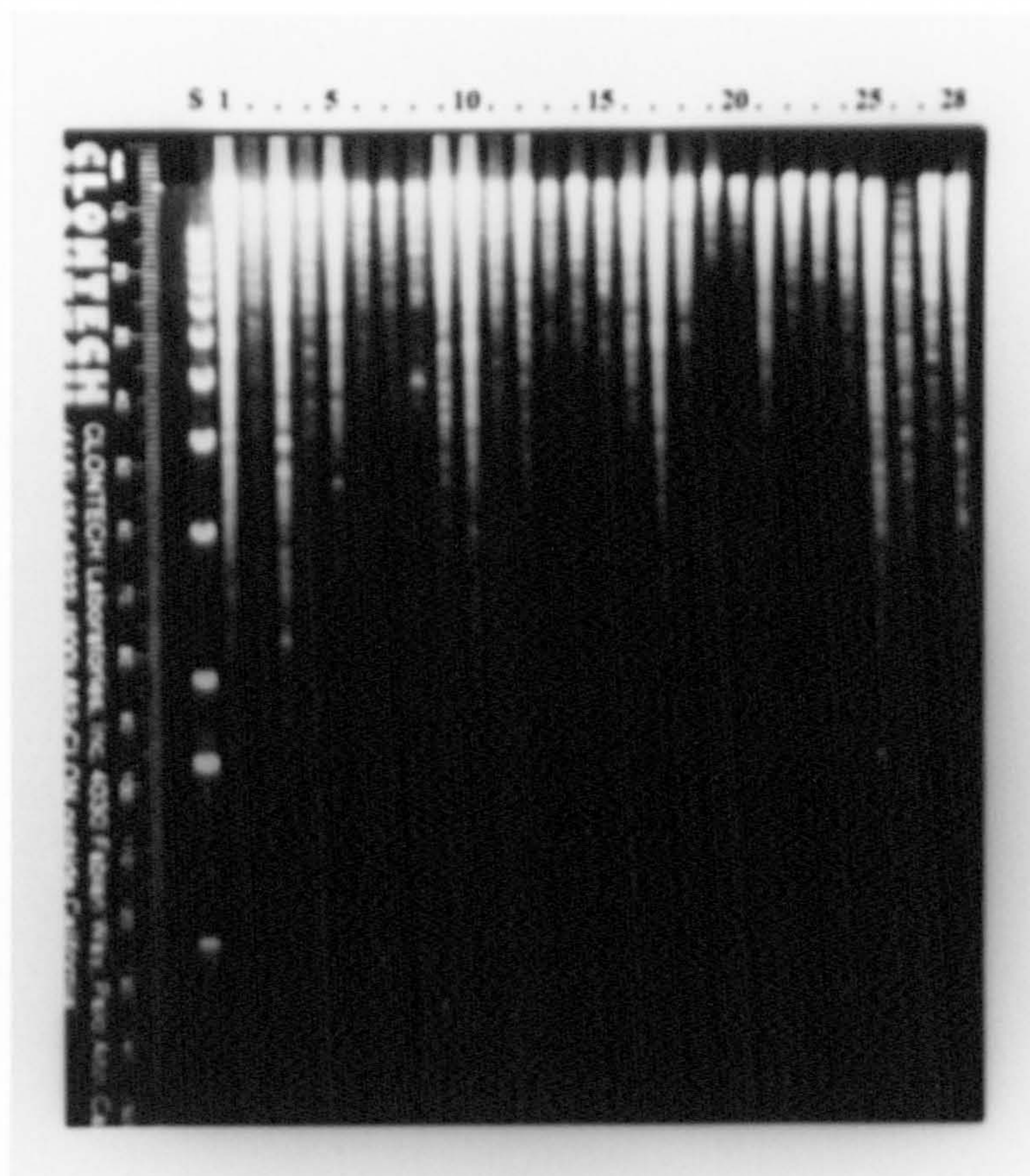


Figure 5.12a Restriction enzyme digests of various bacteria chromosomal DNA: The search for homologues of *rap*. Chromosomal DNAs of various bacteria (listed in the key) digested with *Hind*III.

Key to figure 5.12a and 5.12b

Lane S 1kb ladder

1	<i>Ecc</i> SCRI 112	11	<i>Ecc</i> SCRI 192	21	<i>Sma</i>
2	<i>Ecc</i> SCRI 114	12	<i>Ecc</i> SCRI 193	22	<i>Sbon</i>
3	<i>Ecc</i> SCRI 122	13	<i>Ecc</i> SCRI 198	23	<i>Scin</i>
4	<i>Ecc</i> SCRI 123	14	<i>Ecc</i> ATTn10	24	<i>Escherichia coli</i> DH1
5	<i>Ecc</i> SCRI 124	15	<i>Ecc</i> SCC 3193	25	<i>Yersinia enterocolitica</i>
6	<i>Ecc</i> SCRI 125	16	<i>Eca</i> SCRI 1043	26	<i>Proteus mirabilis</i>
7	<i>Ecc</i> SCRI 132	17	<i>Eca</i> SCRI 39	27	<i>Salmonella typhimurium</i>
8	<i>Ecc</i> SCRI 149	18	<i>Ecb</i> SCRI 479	28	<i>Enterobacter agglomerans</i>
9	<i>Ecc</i> SCRI 166	19	<i>Ech</i> SCRI 1066		
10	<i>Ecc</i> SCRI 172	20	<i>Ech</i> EC16		

Ecc- *Erwinia carotovora* subspecies *carotovora*.; *Eca*- *Erwinia carotovora* subspecies *atroseptica*.; *Ecb*-*Erwinia carotovora* subspecies *betavascularum*.; *Ech*- *Erwinia chrysanthemi*.; *Sma*- *Serratia marcescens* strain ATCC39006; *Sbon*- *Serratia marcescens*, Sutton Bonington strain. *Scin*- *Serratia marcescens* clinical isolate S6.

FIGURE 5.12b

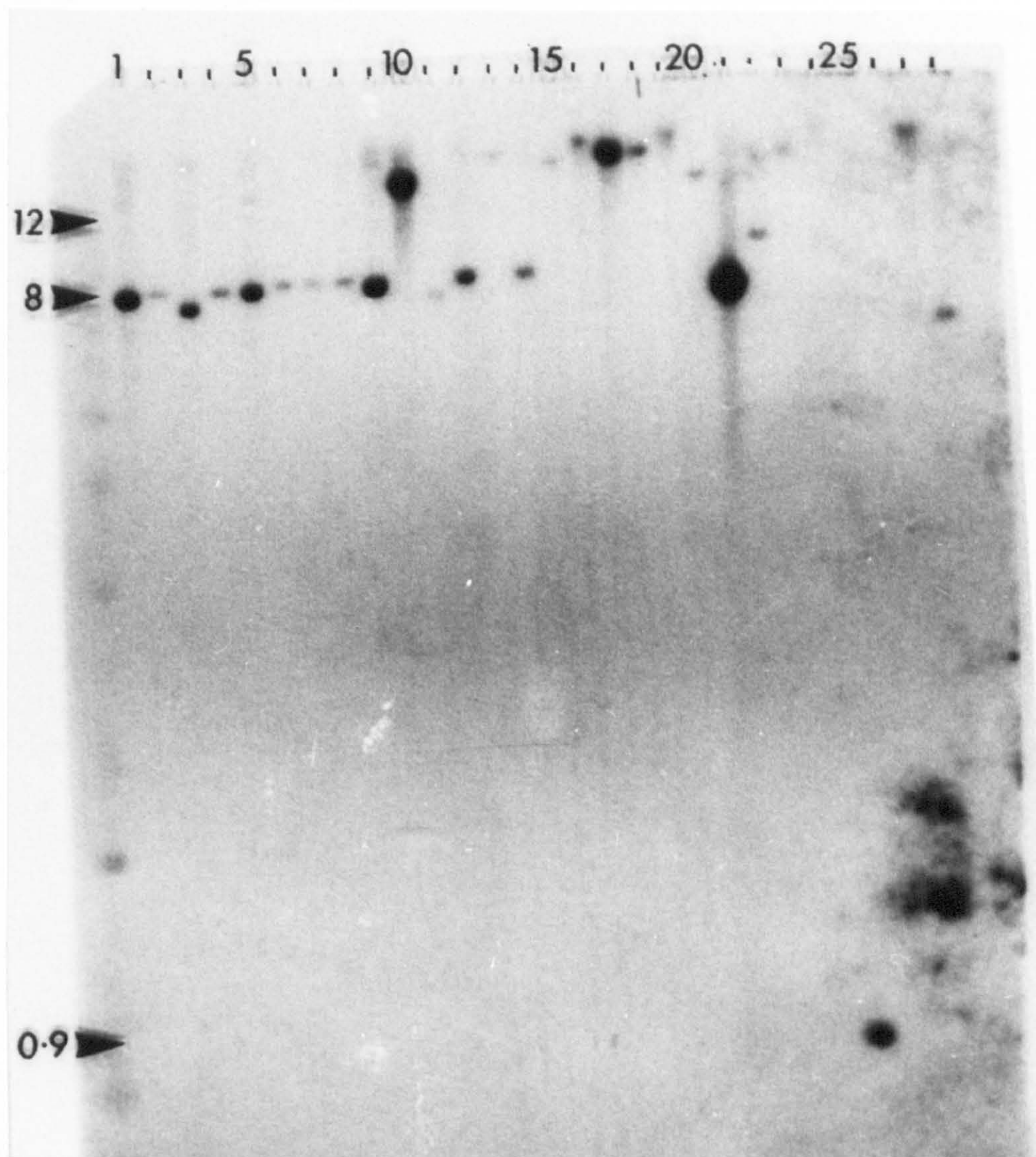


Figure 5.12 Southern blot to detect the distribution of homologues of *rap* in various bacteria.

Samples of chromosomal DNA from various bacterial strains (see key) were digested with *Hind*III and probed with a ^{32}P labelled PCR amplified *rap* fragment. All the bacteria tested exhibited hybridising bands except *E.coli* and *Proteus*. Figures written down the side of the blot represent approximate band sizes and are in kilobases. The hybridising band for the majority of *Ecc* strains was $\sim 8\text{kb}$. The *Y.enterocolitica* hybridising band was very small $\sim 900\text{bp}$. There are two hybridising bands for *Serratia* strain *Sbon*.

approximately 8 kb and for the other species and subspecies the hybridising bands were mainly in excess of 12 kb. The hybridising band for *S.marcescens* was comparable in size to the hybridising bands observed for the majority of the *Ecc* strains and quite distinct from the bands detected for all the other *Serratia* species tested. There were no hybridising bands detectable, under the conditions of this Southern blot, for *E.coli* and *Proteus* (lanes 24 and 26). However there were bands for *Yersinia*, *Salmonella* and *E.agglomerans* in lanes 25, 27 and 28 respectively. The band observed for *Salmonella* DNA was presumably the *rap* probe hybridising to *slyA*.

5.7 HOMOLOGUES OF Rap AND SlyA RETRIEVED FROM THE DATA BASE

The evidence detailed so far cast serious doubts on the conclusions reached by Libby *et al.*, (1994). Rap had a clear role in antibiotic and pigment production in *Serratia* and was distributed widely. During the final stages of this study Dehoux and Cossart, (1995) reported that several known bacterial regulatory proteins shared homology with SlyA (figure 5.13). These included MprA and MarR from *E.coli* and PecS from *Erwinia chrysanthemi*. MprA is a protein which regulates the synthesis of microsins B17 and C7 (Del Castillo *et al.*, 1991). MarR is a repressor of the *marAB* operon responsible for chromosome-mediated multiple antibiotic resistance (Cohen *et al.*, 1993a; Ariza *et al.*, 1994). PecS is a regulatory protein involved in the regulation of pectinase and cellulase production and the production of a extracellular insoluble blue pigment (Reverchon *et al.*, 1994). The identity (similarity in brackets) of these protein regulators with respect to Rap was :- MarR- 24% (56%); PecS- 24% (47%); MprA- 23% (50%).

In addition to these protein homologues, the recent report by Ludwig *et al.*, (1995) identified homologues of SlyA in various *E.coli* strains, *Shigella* and *Citrobacter*. but not in other *Enterobacteriaceae*, *Yersinia*, *Vibrio*, *Pseudomonas* and *Rhizobium* species. The level of homology between Rap and these proteins is summarised in table 6.1.

FIGURE 5.13

	1					50
RapMELPLG	SDLARLVRVW	RALIDHRLKP	
SlyAMKLESPLG	SDLARLVRIW	RALIDHRLKP	
MarRMVNQKK	DRLINEYLSP	
PecS	.MARYLEVSD	IVQQWRNERP	DLDVEPMLVI	GTLSRVSLLI	DRALDKVFSK	
MprA	MDSSFTPIEQ	MLKFRASRHE	DFPYQEILLT	RLCMHMQSKL	LENRNKMLKA	
Cons	-----	-----	-----E--L-	--L-RL----	--L-D--LKP	#
	51					100
Rap	LELTQTHWVT	LHNIHELPPG	Q..SQIQLAK	AIGIEQPSLV	RTLDQLEDKG	
SlyA	LELTQTHWVT	LHNIHQLPDP	Q..SQIQLAK	AIGIEQPSLV	RTLDQLEDKG	
MarR	LDITAAQFKV	LCSIR...CA	ACITPVELKK	VLSVDLGALT	RMLDRLVCKG	
PecS	YKLSAREFDI	LATLRRRGAP	YAYSPSQIVN	ALMINNSTLT	SRLDRLEQAG	
MprA	QGINETLFMA	LITL.ESQEN	HSIQPSELSC	ALGSSRTNAT	RIADELEKRG	
Cons	LELT-T-F--	L--I-----	---SPIQL-K	ALGIE---LT	R-LD-LE-KG	
		*	#	#	#	#* *# *
	101					150
Rap	LITRHICVHD	RRAKRIMLTD	MADPIIQAVN	DVIDQ...TR	SEILNGITPE	
SlyA	LISRQTCASD	RRAKRIKLTE	KAEPLIAEME	EVIHK...TR	GEILAGISSE	
MarR	WVERLPNPND	KRGVLVKLTT	GGAAICEQCH	QLVGQDLHQE	..LTKNLTAD	
PecS	WLRMPPIEGD	RRSVNIQLTD	EGFAL...IN	RVVEEHVENE	RDILSPFSEE	
MprA	WIERRESND	RRCLHLQLTE	KGHEF...LR	EVLPPQHNCL	HQLWSALSTT	
Cons	WI-R-----D	RR---I-LT-	-G--L-----	EV-----	-EIL--LS-E	
	*	* #*	**	#		
	151					180
Rap	EVSELATIIS	RLESNILSLY	EMQS.....			
SlyA	EIELLIKLIA	KLEHNIMELH	SHD.....			
MarR	EVATLEYLLK	KVLP.....			
PecS	EKTHLRALLG	RVEKHLVNNR			
MprA	EKDQLEQITR	KLLSRLDQME	QDGVVLEAMS			
Cons	EV--L--L--	KLE-----L-	-----			
	*	*				

Key - * = all conserved # = 4 out of 5 conserved

Figure 5.13 A line up of Rap and SlyA homologues retrieved from the data base. Multiple alignments were drawn using PILEUP. Positions with at least 3 identical or conserved amino acids appear in the consensus (cons). MprA -Del Castillo *et al.*, (1991). MarR -Cohen *et al.*, (1993); Ariza *et al.*, (1994). PecS - Reverchon *et al.*, (1994). SlyA - Libby *et al.*, (1994). Rap - this study.

5.8 DISCUSSION

The results of the Southern blot, using the *rap* PCR product, were very unexpected: homologues of *rap* were detected in all the bacteria tested with the exception of *E.coli* and *Proteus*. Hybridising bands observed for the wide variety of *Erwinia* species tested suggesting homologues of *rap* are ubiquitous in the erwinias. The size of the restriction fragments observed for the *Ecc* strains was distinct from the bands for the other erwinias tested, with the exception of *Ecc* strain SCRI172, SCRI198 and SCC3193. However strains SCRI172 and SCRI198 previously classified as *Erwinia carotovora* subspecies *carotovora*, have in recent ribotyping studies been shown to be taxonomically distinct from the majority of *Erwinia carotovora* subspecies *carotovora* strains (Holden, 1996).

The hybridising bands for the positive control, *S.marcescens* strain ATCC39006, was comparable to the size of the bands for the *Ecc* strains. The other *Serratia* strains, including the clinical isolate, revealed quite large hybridising bands of about 15-20 kb. There were two bands for the *Sbon* strain, which may mean the *rap* homologue was in multicopy in this strain, or more likely the enzyme cut within the gene.

The band observed for the *Salmonella* DNA was thought to be *slyA* and this would indicate that the Rap PCR probe can detect the homologue in *Salmonella*. There was also a hybridising band detected for *Yersinia* and *Enterobacter*. This was interesting because in addition to *Serratia* these two bacteria were also mentioned in the study of Libby *et al.*, (1994) and Ludwig *et al.*, (1995) as being strains where no such homologues were detected. No hybridising band was detected for *E.coli*. However as the very recent study of Ludwig *et al.*, demonstrates, *E.coli* has a gene encoding a SlyA related protein, which shares 71.1% identity at the protein level with Rap. As the DNA sequence for this gene has not been published we must assume that the identity of this gene is too low to be detected under the conditions used in this Southern blot.

The discovery of protein regulators with significant levels of identity to SlyA (Dehoux and Cossart, 1995) and Rap gave credibility to the idea which was mooted by Libby *et al.*, (1994) but not thought likely, that SlyA was a positive regulator of cryptic *E. coli* strain K-12 haemolysin genes. These homologues were not detected by Libby *et al.*, (1994) or in the initial data base searches of this study. All these protein homologues and the SlyA related proteins recently discovered by Ludwig *et al.*, (1995) are discussed in detail in section 6.5 along with all the homologues of *rap* discovered in this study and so will not be detailed further here.

This Southern blot and the protein homologies clearly illustrate that homologues of *rap* are represented in nearly every bacterium tested including human and animal pathogens in addition to the plant pathogens from the genus *Erwinia*. This breadth in the distribution of *rap* homologues and the level of DNA conservation (dictated by the conditions of the blot) and restriction fragment size for the *Ecc* strains indicates that these genes are highly conserved. This may suggest that homologues of *rap* perform important functions in a wide number of as yet untested bacteria.

To understand further the role performed by Rap in different bacteria it was considered important to direct research into isolating other homologues of the *rap* gene. Historically the focus of research for our laboratory has been the plant pathogen *Erwinia carotovora* subspecies *carotovora*. *Erwinia* strain ATTn10 (also known as GS101), which produces a carbapenem antibiotic identical to that produced by *S. marcescens* strain ATCC39006 (Parker *et al.*, 1982), was chosen for further research to determine what significance a gene required for survival in murine macrophages had in a plant pathogen.

***ISOLATION AND SEQUENCE
ANALYSIS OF THE HOMOLOGUES
OF rap***

6.0 PREFACE

The *rap* gene, shown to be responsible for antibiotic and pigment production in *S.marcescens* (section 5.2), is homologous to *slyA*. SlyA was classified by Libby *et al.*, (1994) as a virulence factor required for the survival of *Salmonella* in murine macrophages. However, SlyA has also been reported to share a high degree of amino acid identity with a wide range of bacterial regulatory proteins (Dehoux and Cossart, 1994) which gave credibility to the idea that SlyA was a regulatory protein and not a virulence factor as had been previously reported. Very recently Ludwig *et al.*, (1995) have shown that SlyA is sufficient to induce haemolytic activity in *E.coli* strain K-12, but is not a cytolysin itself and so SlyA is thought to be a positive regulatory protein (at least in *E.coli*). Southern blot analysis (section 5.6) clearly demonstrated that homologues of *slyA* and *rap* were widely distributed in the erwinias and present in a diverse range of animal and human pathogens. To continue the investigation of these highly conserved homologues of Rap and SlyA, the scope of this study was widened to isolate additional homologues of *rap* from other bacteria and to study the function of these proteins in their respective backgrounds.

Historically the genus *Erwinia* had been the focus of the research in our laboratory, looking at exoenzyme synthesis and secretion and more recently looking at the production of a carbapenem antibiotic, identical in structure to that produced by *S.marcescens*. It was difficult to understand why a plant pathogen should have a homologue of SlyA (as classified by Libby *et al.*, 1994) and so, because *Ecc* was genetically amenable, it was an ideal background to begin investigating the homologues of *rap* denoted *hor* (homologue of *rap*).

6.1 ISOLATION OF *hor*

6.1.1 PREVIOUS WORK

An *Ecc* strain ATCC39048 chromosomal library (Reeves, 1991) was transduced into various mutant strains of *S.marcescens* and *Ecc*, including the

S.marcescens Rap mutant NT5 (Cox, 1995). Two complementing cosmids, pTC51 and pTC52, were isolated from this strain by their ability to simultaneously restore the production of prodigiosin and carbapenem in the Rap mutant, exactly as the *S.marcescens* complementing cosmid pNRT300 had done (section 3.0). Thus it appeared that *Ecc* had a gene(s) which not only hybridised with the *rap* probe (section 5.6) but was also capable of heterogeneric complementation of the *S.marcescens* Rap mutants (T.Cox unpublished results).

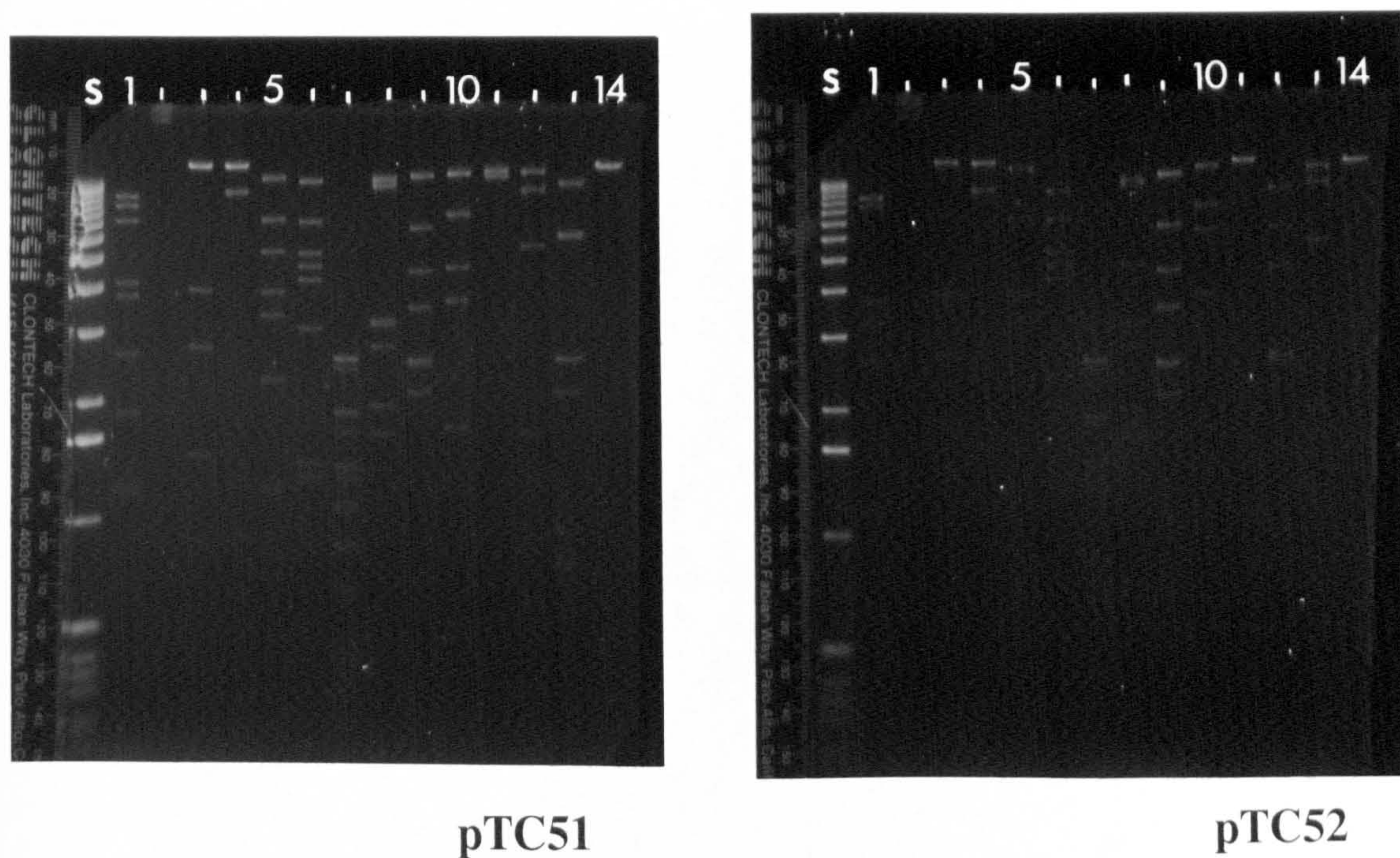
6.1.2 ANALYSIS OF COSMIDS pTC51 AND pTC52

Cosmids pTC51 and pTC52 each contained approximately 30 kb of chromosomal DNA, cloned into the *Bam*HI site of pSF6 (Selveraij *et al.*, 1984). They were both digested with various restriction enzymes to determine if there were any common bands and thus contained overlapping chromosomal DNA fragments. There were very few differences between the restriction fragment banding patterns of the two cosmids (figure 6.1a) and so cosmid pTC51 was chosen for all later experiments.

Erwinia ATTn10 was shown to have a *rap* homologue from the Southern blot described in section 5.6. However, it was not known if this was the same gene as that contained on the complementing clone pTC51. To discover firstly: if pTC51 contained the *Ecc* homologue of *rap* and secondly: if it did, to identify the smallest restriction fragment encoding this gene, the agarose gel containing the various enzyme digests of pTC51 (figure 6.1a) was prepared for a Southern blot and probed with a 474 bp PCR product encoding the *rap* Orf (made using primers Cyto1 and Cyto2, see appendix; labelled with digoxigenin-11-dUTP-DIG).

The autoradiograph (figure 6.1b) revealed there to be only one hybridising band in each of the digests. The smallest observed hybridising bands were from the *Cla*I (0.8 kb) and the *Eco*RV (3.5 kb) digests. It was possible that the *Cla*I fragment contained the whole *hor* gene, assuming *hor* was similar in length to the *rap* gene (460 bp). However, because the DNA flanking the *hor* gene would also be of interest the *Cla*I fragment was targeted for direct cloning into bacteriophage M13mp18 (Messing

FIGURE 6.1a



Key to figures 6.1a and 6.1b		
Lane:	Enzyme	Enzyme
S.	1kb ladder	
1.	<i>Ava</i> II	8. <i>Eco</i> RI
2.	<i>Bcl</i> I	9. <i>Eco</i> RV
3.	<i>Bam</i> HI	10. <i>Hind</i> III
4.	<i>Bgl</i> II	11. <i>Kpn</i> I
5.	<i>Cla</i> I	12. <i>Pvu</i> I
6.	<i>Dra</i> I	13. <i>Sal</i> I
7.	<i>Dde</i> I	14. <i>Sma</i> I

Figure 6.1a A comparison of pTC51 and pTC52 digested with various restriction enzymes. Cosmids pTC51 (left hand side) and pTC52 (right hand side) were digested with various restriction enzymes (see key). The order of enzymes was the same for both of the gels shown.

FIGURE 6.1b



Figure 6.1b Southern blot of pTC51 using a PCR amplified (DIG labelled) *rap* probe.
The letters indicate the positions of the 3.5 kb *EcoRV*(A) and the 0.8 kb *ClaI* (B) hybridising fragments, which were cloned in order to isolate the *Ecc* homologue of *rap* (see text).

and Vieira, 1982) for sequencing, concurrently with the subcloning and eventual sequencing of the 3.5 kb *EcoRV* fragment.

6.1.3 SUBCLONING AND SEQUENCING THE *Ecc* HOMOLOGUE OF *rap (hor)*

Cosmid pTC51 was digested with *ClaI*, the 0.8 kb fragment was purified from an agarose gel, end repaired and ligated into M13mp18 cut with *SmaI*. *E.coli* strain TG1 was transformed with the ligation and white plaques were picked and prepared for sequencing using the M13 universal -40 primer as dictated in the manufacturers instructions (sections 2.8; 2.8.5).

The nucleotide sequence obtained from sequencing several M13 clones revealed this fragment had been cloned in both orientations (data not shown). This allowed the sequence of the whole *ClaI* fragment to be determined without requiring any extra primers (figure 6.3). The sequence of the 0.8 kb *ClaI* fragment (exact size was 874 bp) was translated in six phases. Unfortunately it only encoded part of an Orf, however the predicted gene product from the available sequence (21 amino acids) lined up convincingly with Rap. In order to determine the sequence for the whole Orf the 3.5 kb *EcoRV* fragment was targeted for subcloning and sequencing. The sequence of the *ClaI* fragment was instrumental in the subcloning of the *EcoRV* fragment because a unique *DraI* restriction site was identified (figure 6.3) and this was used to isolate the correct subclone encoding the putative *hor* gene from the *EcoRV* fragment.

The 3.5 kb *EcoRV* fragment was cloned into the *EcoRV* site of the medium copy vector pACYC184 (Chang and Cohen,1978). The ligation products were used to transform *E.coli* strain DH1 and transformants were selected on NBA supplemented with chloramphenicol. To begin subcloning this recombinant plasmid, denoted pNTC35, a simple restriction map was constructed (figure 6.2) in a similar manner to section 4.2.

The 2.4 kb and 1.2 kb *EcoRV* - *SaII* fragments (figure 6.2) of pNTC35, identified from the restriction map, were subcloned into complementary sites in cloning

FIGURE 6.2

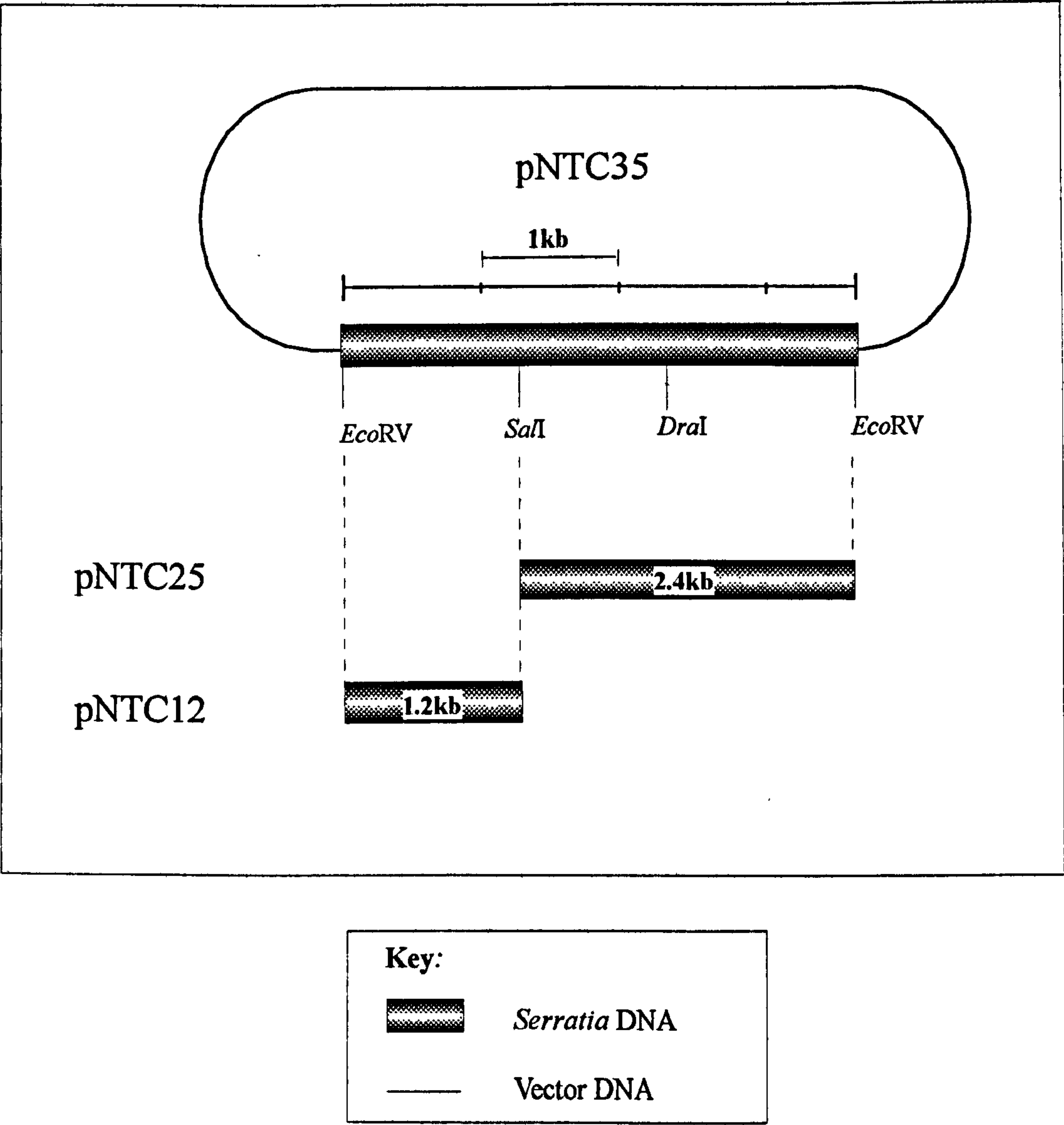


Figure 6.2 A schematic restriction map of pNTC35.
The important restriction sites used to isolate the *Ecc hor* gene are shown. The subclones generated as a result of this map are drawn below with their given names to the left.

vector pACYC184 (Chang and Cohen, 1978). The recombinant plasmids, denoted pNTC25 and pNTC12 respectively, were used to transform *E.coli* strain DH1. Plasmid pNTC25 affected the recipient *E.coli* cells by reducing their growth rate (data not shown). This effect was similar to that caused by the *S.marcescens* plasmid pNRT324 when cloned into *E.coli* (section 4.4).

Plasmids pNTC25 and pNTC12 were cut with restriction enzymes *Cla*I and *Dra*I (data not shown) and run on an agarose gel. In addition to having a single *Dra*I site plasmid pNTC25 also contained an internal 0.8 kb *Cla*I fragment which was identical in size to the *Cla*I fragment that had been sequenced (data not shown). In double enzyme digests of pNTC25, the *Dra*I site was localised to this internal *Cla*I fragment, thereby identifying plasmid pNTC25 as the subclone encoding at least part of the putative *hor* gene.

To determine whether pNTC25 contained a functional *hor* gene, *S.marcescens* *rap* mutant strain NT5 was transformed with this plasmid and transformants were selected on NBA supplemented with chloramphenicol. The recovered transformants had restored pigment (Pig⁺) production and when tested on the *E.coli* strain ESS carbapenem bioassay produced carbapenem (Car⁺), thus indicating that pNTC25 did indeed encode an active *hor* gene.

6.2 SEQUENCE ANALYSIS OF pNTC25

The 2.4 kb *Eco*RV-*Sal*I fragment was excised from pNTC25, cloned into M13mp18 and sequenced using the M13 dideoxynucleotide chain termination method of Sanger *et al.*, (1977) (section 2.8).

Plasmid pNTC25 was found to encode two complete and one partial open reading frame (Orf), which are shown below the sequence in figure 6.3. The Orfs were numbered *orf*1-3, beginning at the 5' end of the displayed sequence. To reduce confusion with the *S.marcescens* genes (of this study) the putative *Ecc* genes were tagged with the suffix 'Ec' unless otherwise stated.

FIGURE 6.3

10 30 50
SalI-----+-----+-----+-----+-----+-----+
GTCGACGCCCCAGTTGTAGGTCAGTTCGATAACTGCGCCTTCGCTCTCTTCGGTATAACC

70 90 110
-----+-----+-----+-----+-----+-----+
GACGAAAGCCAACGTGTATTTGTATCGGTATTCTCGCTGGTGCGCAGCAGCGCATGCCAA

130 150 170
-----+-----+-----+-----+-----+-----+
GGATTTGGGTATAGAAATCGATAGAACGTTGTAAATCGCCAACACGCAGCATGGTGTGAA

190 210 230
-----+-----+-----+-----+-----+-----+
GTAAGCGCATAGTGTCTCGTTAAAAAATGCAACAAAGAGGGAAGTATAGCGTTGTGGCG

250 270 290
-----+-----+-----+-----+-----+-----+
CTACCGAGTCCAAGCTGTTGGGATGATGCTAAAAATGCGGACACTACAGTGAGGTTTGTGT

310 330 350
-----+-----+-----+-----+-----+-----+
TAAACGTACTCTCAGGAGGGGTAAATAGCGCGTTGGGGCAATTTTAGGCTGCCTGACGT

370 390 410
-----+-----+-----+-----+-----+-----+
GTAAAAAATCGTCAGTTATTACAATGGTTACCTAATTATTTTCATTGTGACTGTCCGTT

430 450 470
-----+-----+-----+-----+-----+-----+
TGGGCTTGCCAGCGGTAAAGAACAGGGCTTCAATAAATAGAGTTGTAGCAGGAGGTGTTG

490 510 530
-----+-----+-----+-----+-----+-----+
TGCCAGAGCAACTTGAACCTTTGTTGTCCCTAATCCATGCCGTGGTATTTGTCAGGCGG

550 570 590
-----+-----+-----+-----+-----+-----+
ATGAAGGCGGATATTGTCGCGGTTGCTTTCGCAGTCGTAATGAGCGCTTTAGTTGGGGCC

610 630 650
-----+-----+-----+-----+-----+-----+
AAATGAGTGATGCGCAGAAACAGGATGTGCTGCGTTTGTGTCGGCAGAGAATGAAACGTT

670 690 710
-----+-----+-----+-----+-----+-----+
CACTGCGTTCAGAGAAATCCGATACACCAGCAGAGTCCCGTCAGCCATCGTTGTTCTAAG

730 750 770
-----+-----+-----+-----+-----+-----+
TTATCTTGTCTTAGATGATCTATTTACGTGAAATTTTGTTTAAGCAACGCAGGAGCGTTC

790 810 830
-----+-----+-----+-----+-----+-----+
GATTGAATAAAATAAGCCTGAGATCATTTTTTAAAGACTTTTATTTGATAGAATCCATTT

850 870 890
-----+-----+-----+-----+-----+-----+
CATATGGAGCTATTTTTTACAACAATAAAGTCATTTTTTATTCTTATAAATCAGAGTTGAT


```

          910                      930                      960
-----+-----+-----+-----AGGAGa----orfl2>>-----+
TGCAATCCAGGGATATAACTTAGCGTGCTAACAATAAGGAGAGGTGATGGAATTGCCATT
                               SD           MetGluLeuProLe

          970                      990                      1010
-----+-----+-----+-----ClaI-----+
AGGATCTGATTTAGCCCGTCTGGTGCGCGTATGGCGTGCGCTGGTCGATCATCGATTAAA
uGlySerAspLeuAlaArgLeuValArgValTrpArgAlaLeuValAspHisArgLeuLy

          1030                    1050                    1070
-----+-----+-----+-----+
ACCACTTGAAGTGAAGTCAAGACGCATTGGGTCACGTTGCATAACATATACCATCTACCCCC
sProLeuGluLeuThrGlnThrHisTrpValThrLeuHisAsnIleTyrHisLeuProPr

          1090                    1110                    1130
-----+-----+-----+-----+
AGGGCAGTCGCAGATTCAACTCGCCAAAGCGATAGGTATTGAGCAACCCTCATTAGTCCG
oGlyGlnSerGlnIleGlnLeuAlaLysAlaIleGlyIleGluGlnProSerLeuValAr

          1150                    1170                    1190
-----+-----+-----+-----+
AACACTGGATCAGCTTGAGGAAAAAGGGTTAATCACTCGCCACGTTTGTGCGCACGATCG
gThrLeuAspGlnLeuGluGluLysGlyLeuIleThrArgHisValCysAlaHisAspAr

          1210                    1230                    1250
-----+-----+-----+-----+
TCGGGCAAAACGTATTATGCTGACCGAATCAGCAGAGCCAATCATACAGGCAGTCAATGG
gArgAlaLysArgIleMetLeuThrGluSerAlaGluProIleIleGlnAlaValAsnGl

          1270                    1290                    1310
-----+-----+-----+-----+
TGTAATTAGCCATACACGTAGTGAAGTCTTATTTGGTATTACGCCTGAGCAAGTGGATGA
yValIleSerHisThrArgSerGluValLeuPheGlyIleThrProGluGlnValAspGl

          1330                    1350                    1370
-----+-----+-----+-----+
ATTAGCGTTGCTGGTTTCGCGTCTTGAGAAAAATATATTGGCATTACATGAGAATCAAGC
uLeuAlaLeuLeuValSerArgLeuGluLysAsnIleLeuAlaLeuHisGluAsnGlnAl

          1390                    1410                    1430
-----+-----+-----+-----ClaI
GTAGCTAAATTTGGGTTACGCAGAGGGAGAATTAAATCCCGGCAAGATTACCGGTATCGA
aEnd

          1450                    1470                    1490
-----+-----+-----+-----+
TTATTGGTTATTTATTCCGGGAGTTGTTATTCGTTGACTCGGCCATCTGCGGCGATCGTC

          1510                    1530                    1550
-----+-----+-----+-----+
ACACAGCATTACCGTGGGGAAACGGTAATACTGCGGCCATTGCTGGCCATAGCAACGCGT
EndArgProSerValThrIleSerArgGlyAsnSerAlaMetAlaValArgG

          1570                    1590                    1610
-----+-----+-----+-----+
TGACCTGCGCTGAATTTTCGTATCGCCTTGTCTGTACCACCATGATGGTACTGCCATCA
lnGlyAlaSerPheLysThrAspGlyGlnLysGlnValValMetIleThrSerGlyAspA

          1630                    1650                    1670
-----+-----+-----+-----+
TCACGACGAATTTCCAGTTCTACGCCCTGTGTGCGGTTTAGCGCACCTGTGGCGCTTTGA
spArgArgIleGluLeuGluValGlyGlnThrArgAsnLeuAlaGlyThrAlaSerGlnG

```


1690 1710 1730
 -----+-----+-----+-----+
 CCCGCTACCCACCGGCTACGGCGCCAGCTGCTGTCGCCAGGCTACGACCGGAGCCGCCA
 lyAlaValGlyGlyAlaValAlaGlyAlaAlaThrAlaLeuSerArgGlySerGlyGlyG

1750 1770 1790
 -----+-----+-----+-----+
 CCGATAGTATTACCCAGGAACCCCCCAGAACAGCACCGCCGAGCGCGCCGATCACGTTA
 lyIleThrAsnGlyLeuPheGlyGlyLeuValAlaGlyGlyLeuAlaGlyIleValAsnS

1810 1830 1850
 -----+-----+-----+-----+
 GAATCTTCTCCCGCCTGAATCTGAACCGGGCGCGTAGAAACAATGGTACCGTAGGTCACG
 erAspGluGlyAlaGlnIleGlnValProArgThrSerValIleThrGlyTyrThrValT

1870 1890 1910
 -----+-----+-----+-----+
 GTCTGCACTTGTTTAGCTTCGGATGCGCTGTAAACATCACCTGAAAGCGTACTGGTATTA
 hrGlnValGlnLysAlaGluSerAlaSerTyrValAspGlySerLeuThrSerThrAsnA

1930 1950
 -----+-----+-----+-----+
 GCACAACCAGCCAGCGTGATACCAGCAAGGGTAACCACAAGTAAACGCTTCATCATAATA
 laCysGlyAlaLeuThrIleGlyAlaLeuThrValValLeuLeuArgLysMetMet
 ↑

2010 2030
 -----+-----+-----+-----+
 CAAA**CTCCT**TAATCGCGAATATGTCGGGCTGGCTCAGCTCGGCCGACGCAGTGTTATACC
 SD

2050 2070 2090
 -----+-----+-----+-----+
 AATAAATTATGGTCTGGCTCAGTGTAGCATGCCACTCTTTTACTCTTTTTTTCAGAATA

2110 2130 2150
 -----+-----+-----+-----+
 TAATAGTGCGCTTAAATTGCGTATAAACAGAGGAATAAATAGTACGAGAAAGGCATTTTTT

2170 2190 2210
 -----+-----+-----+-----+
 GTAGCAAATATTATCAAAAATCGATATTTGGTTAAAACCTAACCCGTGTGGCAAAAACCTG

2230 2250 2270
 -----+-----+-----+-----+
 TAAAAAATTTAATAATTCAGTGTGTTAACGTGCTACTTTTACTCTCAACGTAACGGTTGT

2290 2310 2340
 -----+-----+-----+-----+
 TATCGGTGTTCTCTTTTATTTTACAGGGGCAA**AGG**CTATATA**ATG**AGATCAGGCAGATA
 SD MetArgSerGlyArgTy

orf2_{Ec}
 orf3'_{Ec}

2350 2370 2390
 -----+-----+-----+-----+
 TATTGGCGTAATGTCCGGCACCAGCCTTGATGGTGTGGATGTTGTGCTAGCCGCGATTGA
 rIleGlyValMetSerGlyThrSerLeuAspGlyValAspValValLeuAlaAlaIleAs
 2410 2430 2452
 -----+-----+-----+-----+**EcoRV**
 CGAACATACGGTTGCTCAGCAGGCCAGCTACTGTCACCCGATACCGCAGGAT
 pGluHisThrValAlaGlnGlnAlaSerTyrCysHisProIleProGlnAsp

Figure 6.3 The 2452 nucleotide sequence of the *Sall*-*EcoRV* fragment of pNTC25.

The translation of the sequence is written in **blue** below the nucleotide sequence. The initiation codons have been emboldened and the termination codons are marked by an 'End' in the protein sequence below. The ribosome binding sites (**red; SD-Shine & Dalgarno**) are marked in coloured type. The position of the *Cla*I fragment sequenced directly from M13 using the universal primer has been merged into the sequence of pNTC25 between positions 140-1011. The unique *Dra*I site used to isolate the *hor* gene is marked at position 810. The lipoprotein cleavage site for Pcp_{Ec} is marked with an arrow at position 1924.

FIGURE 6.4

	1					50
Rap	MELPLGSD	LARLVRVWRA	LIDHRLKPLE	LTQTHWVTLH	NIHELPPGQS	
Orf1 _{Ec}	MELPLGSD	LARLVRVWRA	LVDHRLKPLE	LTQTHWVTLH	NIYHLPPGQS	
SlyA	MKLESPLGSD	LARLVRIWRA	LIDHRLKPLE	LTQTHWVTLH	NIHQLPPDQS	
Con	ME-PLGSD	LARLVRVWRA	LIDHRLKPLE	LTQTHWVTLH	NI--LPP-QS	
	51					100
Rap	QIQLAKAIGI	EQPSLVRTL	QLEDKGLITR	HICVHDDRRAK	RIMLTDMADP	
Orf1 _{Ec}	QIQLAKAIGI	EQPSLVRTL	QLEEKGLITR	HVCAHDDRRAK	RIMLTESAEP	
SlyA	QIQLAKAIGI	EQPSLVRTL	QLEDKGLISR	QTCASDDRRAK	RIKLTEKAEP	
Con	QIQLAKAIGI	EQPSLVRTL	QLEDKGLI-R	--C--DDRRAK	RI-LTE-AEP	
	101					147
Rap	IIQAVNDVID	QTRSEILNGI	TPEEVSELAT	IISRLESNIL	SLYEMQS*	
Orf1 _{Ec}	IIQAVNGVIS	HTRSEVLFGI	TPEQVDELAL	LVSRLKKNIL	ALHENQA*	
SlyA	LIAEMEEVIH	KTRGEILAGI	SSEEIELLIK	LIACLEHNIM	ELHSHD*	
Con	-I-----VI-	-TR-EIL-GI	--E-V--L--	-I--LE-NIL	-L-----	

Figure 6.4 Multiple alignment of Rap, Orf1_{Ec} and SlyA.

Rap (*Serratia marcescens*; regulation of antibiotic and pigment production; this study) Orf1_{Ec} (*Erwinia carotovora subsp carotovora*; this study) and SlyA (*Salmonella typhimurium*; Salmolysin; Libby *et al.*, 1994); Accession number UO3842 GenBank). The consensus is marked in **blue** showing a minimum of 3 identical or conserved amino acids.

6.2.1 ORF1_{Ec}

The Orf1_{Ec} was 435 nucleotides long (position 947-1384) and was predicted to encode a protein of 145 amino acids with a calculated molecular mass of 16416 Da and a pI of 7.29. The ATG initiation codon was preceded by a putative ribosome binding sequence 4 bp upstream (AGGAGA), which agreed well with the consensus (AGGAGG; Shine and Dalgarno, 1974).

Database searches identified SlyA as the protein which shared the highest level of homology with Orf1_{Ec}. As might be expected from the homology with SlyA, a line-up of Rap and Orf1_{Ec} also showed a pronounced level of amino acid identity, shown in figure 6.4.

The multiple alignment shows Orf1_{Ec} to be homologous to SlyA with an amino acid identity of 71%. Orf1_{Ec} shares a higher level of identity with Rap (83% at the amino acid level) and is the same length as Rap. The level of identity between these three proteins indicated that *orf1_{Ec}* was the *Ecc* homologue of *rap* and so was denoted the *hor* gene.

The nucleotide sequence of pNTC25 was lined up with that of pNRT324 (the *rap* containing plasmid from *S.marcescens*) and the sequences were found to have an identity of 70% over their entire length (data not shown). DNA similarity between the coding regions was lower than within the Orfs. However there was a region of DNA just upstream of *hor* (*orf1_{Ec}*) which was highly conserved with the equivalent region of *rap* (figure 6.5). This DNA displayed several motifs which could potentially act as an operator region for the binding of an activator or repressor. Although with the data available there is no direct evidence for this as yet.

The hydropathy profile of Hor (Orf1_{Ec}), shown in figure 6.6, was found to be very similar to that of SlyA and Rap (figure 4.7), with no obvious transmembrane domains. The PSORT prediction was that this was a cytoplasmic protein consistent with the lack of an export signal.

FIGURE 6.5



Figure 6.5 The conserved region of DNA upstream of the *rap* and *hor* open reading frames. Sequence alignment was performed using BESTFIT on UWGCG. Nucleotide identity is marked with a vertical line. The *rap* sequence is shown above the *hor* sequence and the ATG translational start codons have been emboldened and the putative ribosome binding sites are marked in red (SD). The palindromic sequences are marked by green chevrons and are labelled 1-4. The position of the PCR primer **CyclCytol**, designed to this conserved region of *rap* and *hor* is marked and was important for later experiments.

FIGURE 6.6

The *Orf1_{Ec}* was 405 nucleotides in length (position 1974-1569), encoding a predicted protein of 135 amino acids with a calculated molecular mass of 15443 Da and a pI value of 10.52. The *Orf* had two possible ATG translational start codons, adjacent to each other and both in the same reading frame, the initiation codon at the more favourable distance of 8 by downstream of the possible ribosome binding site, has been highlighted in figure 6.3. The putative ribosome binding site (AGGAGT) conformed well with the consensus.

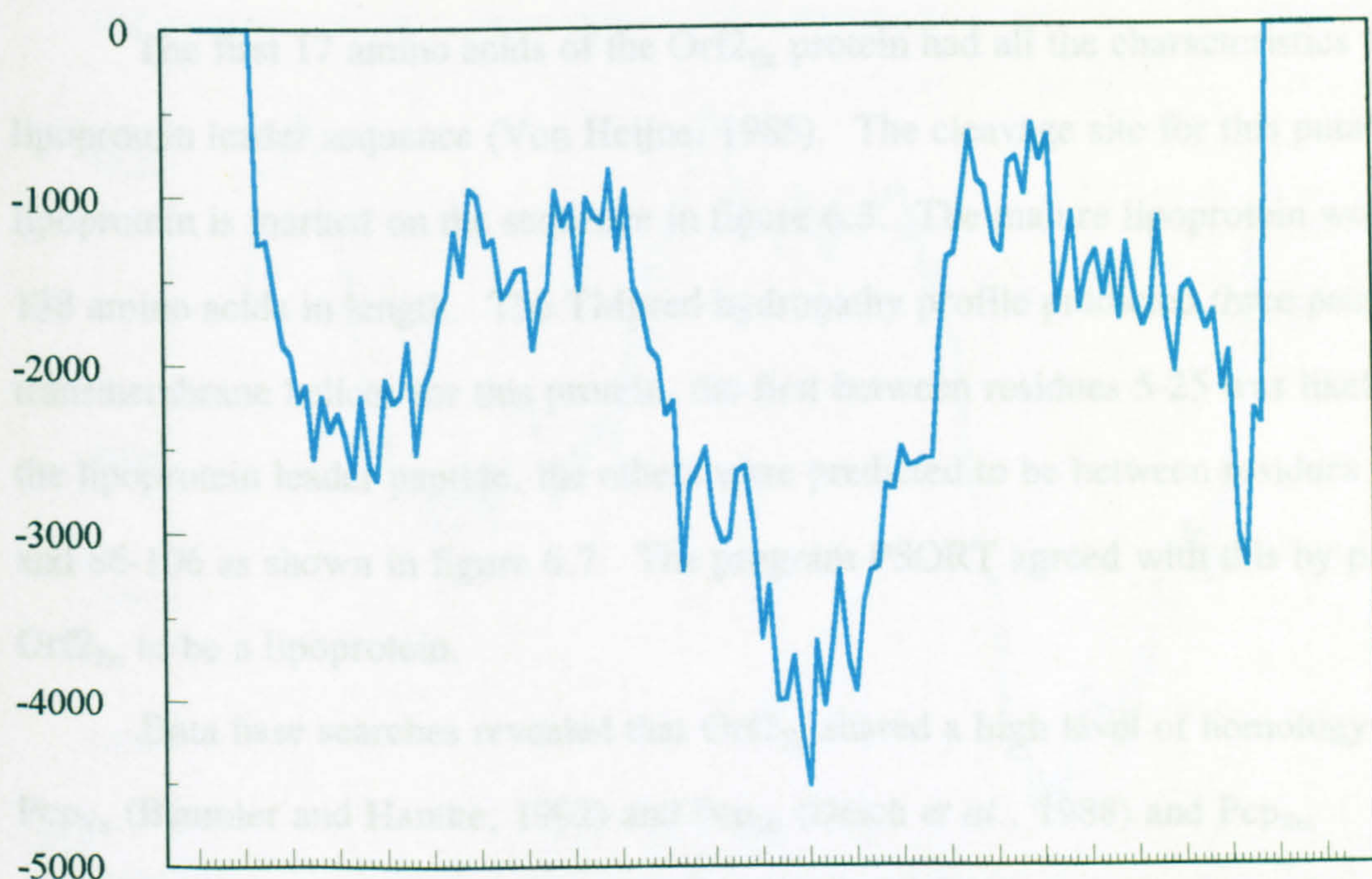


Figure 6.6 The predicted hydropathy profile of Hor (*Orf1_{Ec}*) drawn using TMpred.

The window for this program was set at 17 residues, scores in excess of 500 are considered to be significant in representing a membrane spanning domain. The prediction made from these data was that there were no likely transmembrane domain.

Catalase localisation of lipoproteins has been extensively studied (Grange *et al.*, 1982; Yerraguchi *et al.*, 1983), and the presence of a negatively charged aspartate moiety at position +2 or +3 targets a lipoprotein to the inner membrane. The absence

6.2.2 ORF2_{Ec}

The Orf2_{Ec} was 465 nucleotides in length (position 1974-1509), encoding a predicted protein of 155 amino acids with a calculated molecular mass of 15443 Da and a pI value of 10.52. The Orf had two possible ATG translational start codons, adjacent to each other and both in the same reading frame, the initiation codon at the more favourable distance of 8 bp downstream of the possible ribosome binding site, has been highlighted in figure 6.3. The putative ribosome binding site (AGGAGT) conformed well with the consensus.

The first 17 amino acids of the Orf2_{Ec} protein had all the characteristics of a lipoprotein leader sequence (Von Heijne, 1985). The cleavage site for this putative lipoprotein is marked on the sequence in figure 6.3. The mature lipoprotein would be 138 amino acids in length. The TMPred hydropathy profile predicted three possible transmembrane helices for this protein, the first between residues 5-25 was likely to be the lipoprotein leader peptide, the others were predicted to be between residues 60-81 and 86-106 as shown in figure 6.7. The program PSORT agreed with this by predicting Orf2_{Ec} to be a lipoprotein.

Data base searches revealed that Orf2_{Ec} shared a high level of homology with Pcp_{Ye} (Baumler and Hantke, 1992) and Pcp_{Hi} (Deich *et al.*, 1988) and Pcp_{Sm} (*S. marcescens*; this study) as shown in figure 6.8. These lipoproteins have been discussed previously in section 4.6.2.3. The amino acid identity of Orf2_{Ec} with Pcp_{Sm} was 84%, with Pcp_{Ye} it was 72% and with Pcp_{Hi} it was 47%. Orf2_{Ec} also shared 78% amino acid identity to SlyB a lipoprotein from *Salmonella typhimurium* recently found to be encoded by a gene downstream of *slyA* (figure 6.11; Ludwig *et al.*, 1995). No other proteins were found to share significant levels of homology with Orf2_{Ec} and therefore Orf2_{Ec} was designated Pcp_{Ec}.

Cellular localisation of lipoproteins has been extensively studied (Inouye *et al.*, 1982; Yamaguchi *et al.*, 1988), and the presence of a negatively charged aspartate moiety at position +2 or +3 targets a lipoprotein to the inner membrane. The absence

FIGURE 6.7

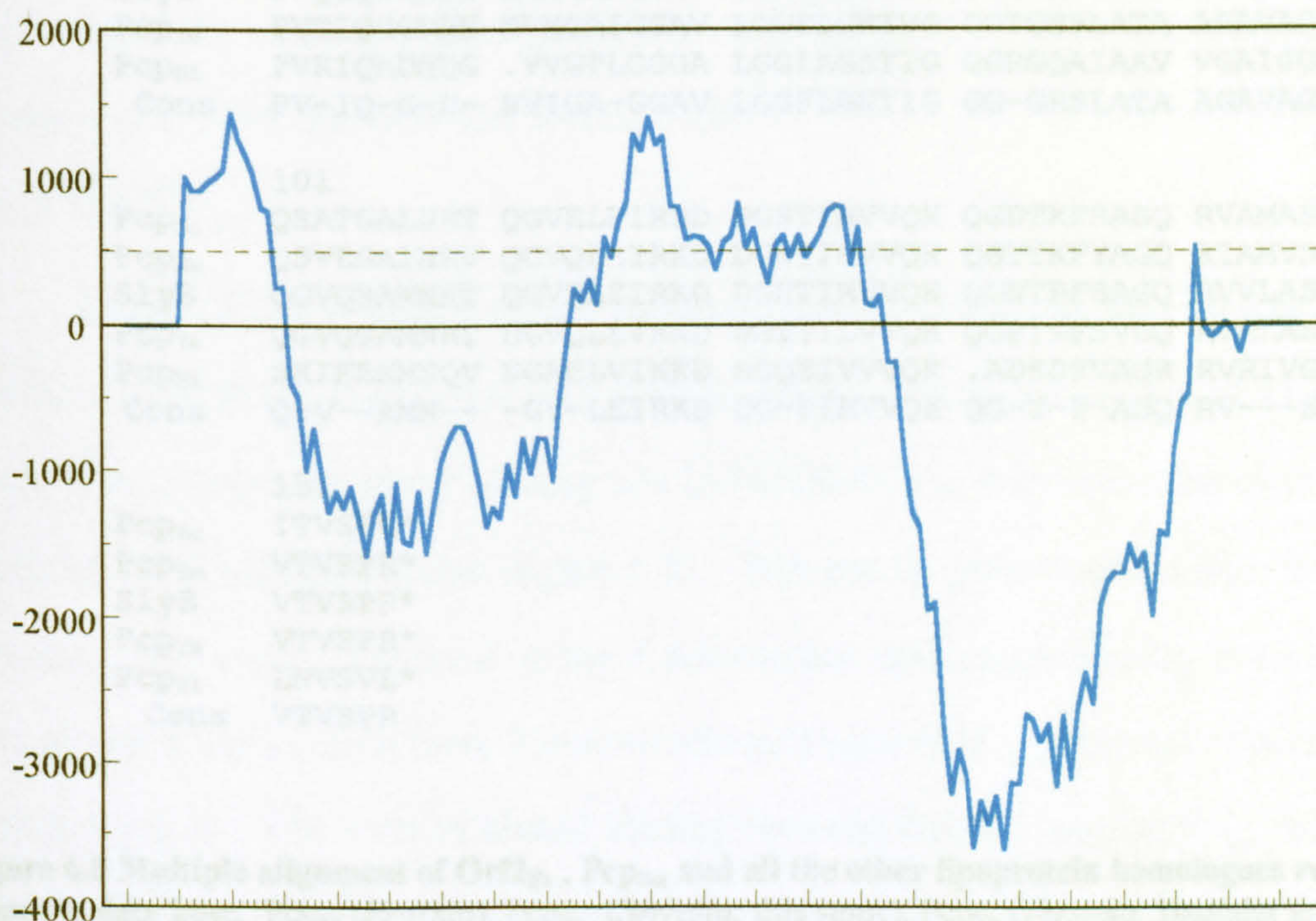


Figure 6.7 The predicted hydropathy profile of Orf2_{Ec} drawn using TMpred.

The window for this program was set at 17 residues and scores in excess of 500 are considered to be significant in representing a membrane spanning domain. The prediction made from these data was that there were three possible transmembrane domains between residues 5-25, 60-81 and 86-106.

FIGURE 6.8

	1				50
Pcp _{Ec}	MMKRLLVVT.	LAGITLAGCA	NTSTLSGDVY	SASEAKQVQT	VTYGTIVSTR
Pcp _{Sm}	MMKYFLVIT.	LASITLVGCA	NTSTLSGDVY	SASDAKQVQT	VTYGTIISTR
SlyB	MIKRVLAVS.	LMGLSLAGCV	NNDSLSGDVY	TASEAKQVQN	VTYGTIVNVR
Pcp _{Ye}	MIKPLIAVA.	IAAVTLTGCA	NNNTLSGDVF	SASQAKQVQT	VTYGTLLSVR
Pcp _{Hi}	MKKTNMALAL	LVAFSVTGCA	NTDIFSGDVY	SASQAKEARS	ITYGTIVSVR
Cons	M-K--L-V--	L-----L-GCA	N---LSGDVY	SAS-AKQVQ-	VTYGTIVS-R
	51				100
Pcp _{Ec}	PVQIQAGEDS	NVIGALGGAV	LGGFLGNTIG	GGSGRSLATA	AGAVAGGVAG
Pcp _{Sm}	PVQIQAGEEN	NIIGALGGAV	LGGFLGNTVG	GGTGRSLATA	AGAVAGGVAG
SlyB	PVQIQGGDDS	NVIGAIGGAV	LGGFLGNTIG	GGTGRSLATA	AGAVAGGVAG
Pcp _{Ye}	PVTIQGGDDN	NVMGAIGGAV	LGGFLGNTVG	GGTGRSLATA	AGAVAGGMAG
Pcp _{Hi}	PVKIQADNQG	.VVGTLGGGA	LGGIAGSTIG	GGRGQAIAAV	VGAIGGAIAG
Cons	PV-IQ-G-D-	NVIGA-GGAV	LGGFLGNTIG	GG-GRSLATA	AGAVAGGVAG
	101				150
Pcp _{Ec}	QSATGALNRT	QGVELEIRRD	DGSTIMVVQK	QGDTKFSAGQ	RVAMASNGRS
Pcp _{Sm}	QSVEGAINRV	QGVQLEIRKD	DGNTIMVVQK	QGTTKFYAGQ	RIAMVSDGRS
SlyB	QGVQSAMNKT	QGVELEIRKD	DGNTIMVVQK	QGNTRFSAGQ	RVVLASNGSQ
Pcp _{Ye}	QGVQGAMNRT	DGVQLEVRKD	DGTTILVVQK	QGPTRFSVGQ	RVMLASSGST
Pcp _{Hi}	SKIEEKMSQV	NGAELVIKKD	DGQEIVVVQK	.ADSSFVAGR	RVRIVGGGSS
Cons	Q-V--AMN--	-GV-LEIRKD	DG-TIMVVQK	QG-T-F-AGQ	RV---S-G--
	151				
Pcp _{Ec}	ITVSPR*				
Pcp _{Sm}	VTVSPR*				
SlyB	VTVSPR*				
Pcp _{Ye}	VTVSPR*				
Pcp _{Hi}	LNVSVL*				
Cons	VTVSPR				

Figure 6.8 Multiple alignment of Orf2_{Ec}, Pcp_{Sm} and all the other lipoprotein homologues retrieved from the data base. Pcp_{Ec} (*Erwinia*), Pcp_{Sm} (*Serratia*; this study), Pcp_{Ye} (*Yersinia*; Baumler and Hantke, 1992), Pcp_{Hi} (*Haemophilus*; Diech *et al.*, 1988) and SlyB (*Salmonella typhimurium*; Ludwig *et al.*, 1995). Alignments were obtained using PILEUP on UWGCG. Positions with at least 4 identical or a conserved amino acids are represented in the consensus.

of a negatively charged residue at this position leads to the translocation of the lipoprotein to the outer membrane. An example of an inner membrane lipoprotein is Lipoprotein-28 of *E.coli* and of an outer membrane lipoprotein is PAL from *H.influenzae* (figure 6.9).

The uncharged residues at position +2 and +3 following the signal peptide cleavage site for Pcp_{Ec} and the *S.marcescens* homologue Pcp_{Sm}, indicated that both of these lipoproteins would be directed to the outer membrane. This is consistent with the fact that the Pcp homologues from *H.influenzae* and *Y.enterocolitica*, which share identical residues at these positions, are known to be localised to the outer membrane (Diech *et al.*, 1988; Baumler and Hantke, 1992).

6.2.3 ORF3'_{Ec}

The Orf3'_{Ec} was a partial open reading frame of 129 nucleotides in length (position 2322-2452), which was predicted to encode a truncated protein of 43 amino acids. The putative ribosome binding site (AGGCTA) was 4 bp upstream of the predicted ATG initiation codon (figure 6.3). This partial gene shared a high level of homology with *orfX'*_{Sm} encoded on the *S.marcescens rap* complementing cosmid and a gene of unknown function from *Y.enterocolitica* (figure 6.10 ; previously discussed in section 4.6.2.4). The level of shared identity between Orf3'_{Ec} and OrfX'_{Sm} was 95% and with Orf1_{Ye} was 88% (percentages relate to shared identity over the limited sequence available; Baumler and Hantke, 1992). No other proteins with significant levels of identity were retrieved from the data base and so Orf3'_{Ec} was designated OrfX'_{Ec}.

6.3 DISCUSSION

The plasmids pTC51 and pTC52 contained overlapping DNA fragments and so further research was restricted to pTC51. The putative *hor* gene was located by Southern blot to a 0.8 kb *ClaI* and a 3.5 kb *EcoRV* (pNTC35) fragment. The *ClaI* fragment was cloned directly into M13mp18 and the sequence derived from it was

FIGURE 6.9

↓

Lipoprotein-28 (<i>E.coli</i>)	MKLTTHHLRTGAALLLAGILLAG	<u>CD</u> QSSS...
PAL (<i>E.coli</i>)	MKATKLVLGAVILGSTLLAG	CSSNAK...
PAL (<i>H.influenzae</i>)	MNKFVKSLLVAGSVAALAA	CSSSNN...
Pcp _{Hi} (<i>H.influenzae</i>)	MKKTNMALALLVAFSVTG	CANTDI...
Pcp _{Ye} (<i>Y.enterocolitica</i>)	MIKPLIAVAIAAVTLTG	CANNNT...
SlyB (<i>S.typhimurium</i>)	MIKRVLAVSLMGLSLAG	CVNND...
Pcp _{Sm} (<i>S.marcescens</i>)	MMKYFLVITLASITLVG	CANTST...
Pcp _{Ec} (<i>E.c.carotovora</i>)	MMKRLLVVTLAGITLAG	CANTST...

Figure 6.9 A comparison of the signal sequences and cleavage sites of selected lipoproteins from Gram negative bacteria. Taken from Yamaguchi *et al.*,(1988): Lipoproteins of selected Gram negative bacteria are lined up at the cysteine (C) cleavage site, position +1. Lipoprotein-28, Yu *et al.*, (1986); *E.coli* PAL, Chen and Henning, (1987); *H.influenzae* Pcp_{Hi} and PAL, Diech *et al.*, (1988); *Y.enterocolitica* Pcp_{Ye}, Baumler and Hantke, (1992); *S.typhimurium* SlyB, Ludwig *et al.*, (1995); Pcp_{Ec} and Pcp_{Sm} (this study). The proteolytic cleavage site is marked by an arrow. The negatively charged aspartate residue that directs Lipoprotein-28 to the inner membrane is underlined at position +2 (see text). All the other lipoproteins depicted here are localised in the outermembrane.

FIGURE 6.10

	1					50
Orf3' _{Ec}MRS	GRYIGVMSGT	SLDGVDVVLA	AIDEHTVAQQ	ASYCHPIPQD	
OrfX' _{Sm}MKS	GRYIGVMSGT	SLDGIDVVLA	AIDEYTVAQQ	ASYCHPIPQS	
Orf1 _{Ye}	MGNKVIAMKS	GRFIGVMSGT	SLDGIDVVLA	AIDERMVAQQ	ASYCHPMPLQ	
Cons	-----MKS-GR-IGVMSGT	SLDG-DVVLA	AIDE--VAQQ	ASYCHP-P--		
	51					100
Orf3' _{Ec}	
OrfX' _{Sm}	IRLAILSMCQ	GQPVTLSALG	HLDTSSVFCL	TEAVLALLKQ	SGISASDI	
Orf1 _{Ye}	LKKDILGMCQ	GQSTTL SAVG	KLESQLGILF	AEAVLALLAK	AGLTAQDITA...	
Cons	----IL-MCQ	GQ--TL SA-G	-L-----	-EAVLALL--	-G--A-DI--	

Figure 6.10 Multiple alignment of Orf3'_{Ec}, OrfX'_{Sm} and Orf1_{Ye}. OrfX'_{Ec} (*Ecc*), OrfX'_{Sm} (*S.marcescens*) and Orf1_{Ye} (*Yersinia*, Baumler and Hantke, 1992). The consensus (blue) shows identical amino acids only.

instrumental in the subcloning of pNTC35. Plasmid pNTC35 was subcloned down to a 2.5 kb *EcoRV-SalI* fragment (pNTC25) which was sequenced revealing two complete and one partial open reading frame.

All of the Orfs carried on pNTC25 shared a striking level of homology with genes discovered on plasmid pNRT324 from *S.marcescens*. Therefore much of the detail concerning the protein homologues of the genes encoded on pNTC25 has already been discussed in chapter 4 and will not be repeated here.

The first Orf encoded on pNTC25 was similar to SlyA from *S.typhimurium* and to Rap from *S.marcescens* (sections 4.6.2.2 and 4.7). Therefore the gene identified by Southern blot (section 5.6) as a homologue of *rap* had been successfully cloned and was named the *hor* gene. The level of amino acid conservation between Hor, Rap and SlyA declines towards the C-terminus and one possible explanation for this is that the functional domains of these proteins are located in the N-terminus. It is interesting to note in this regard that the *Serratia* Rap mutants were complemented by the *Ecc hor* gene despite the C-terminal divergence between these proteins.

Orf2_{Ec} was very similar to Pcp_{Sm}, Pcp_{Hi}, Pcp_{Ye} and SlyB (sections 4.6.2.3 and 4.7) and so was designated Pcp_{Ec}. Pcp_{Ec} had a characteristic lipoprotein leader peptide; the amino acid sequence of which led to the prediction that Pcp_{Ec} would be directed to the outer membrane of the cell. The level of conservation of the Pcp homologues must imply that they perform an important function in this diverse range of bacteria.

Orf3'_{Ec} was found to be similar to OrfX'_{Sm} of *S.marcescens* (this study) and Orf1_{Ye} from *Y.enterocolitica* (sections 4.6.2.4 and 4.7). This Orf was denoted *orfX'*_{Ec}, the function of it's gene product and homologues remains unknown.

Perhaps the most interesting observation made from the sequence of pNTC25, was the conserved gene arrangement between *hor*, *pcpEc* and *orfX'*_{Ec} and their homologues in *S.marcescens*, *Y.enterocolitica* and the recently published *S.typhimurium* sequence (Ludwig *et al.*, 1995; illustrated in figure 6.11). An additional Southern blot (data not shown) probed with a PCR amplified *pcpSm* DNA probe was used to identify

FIGURE 6.11.

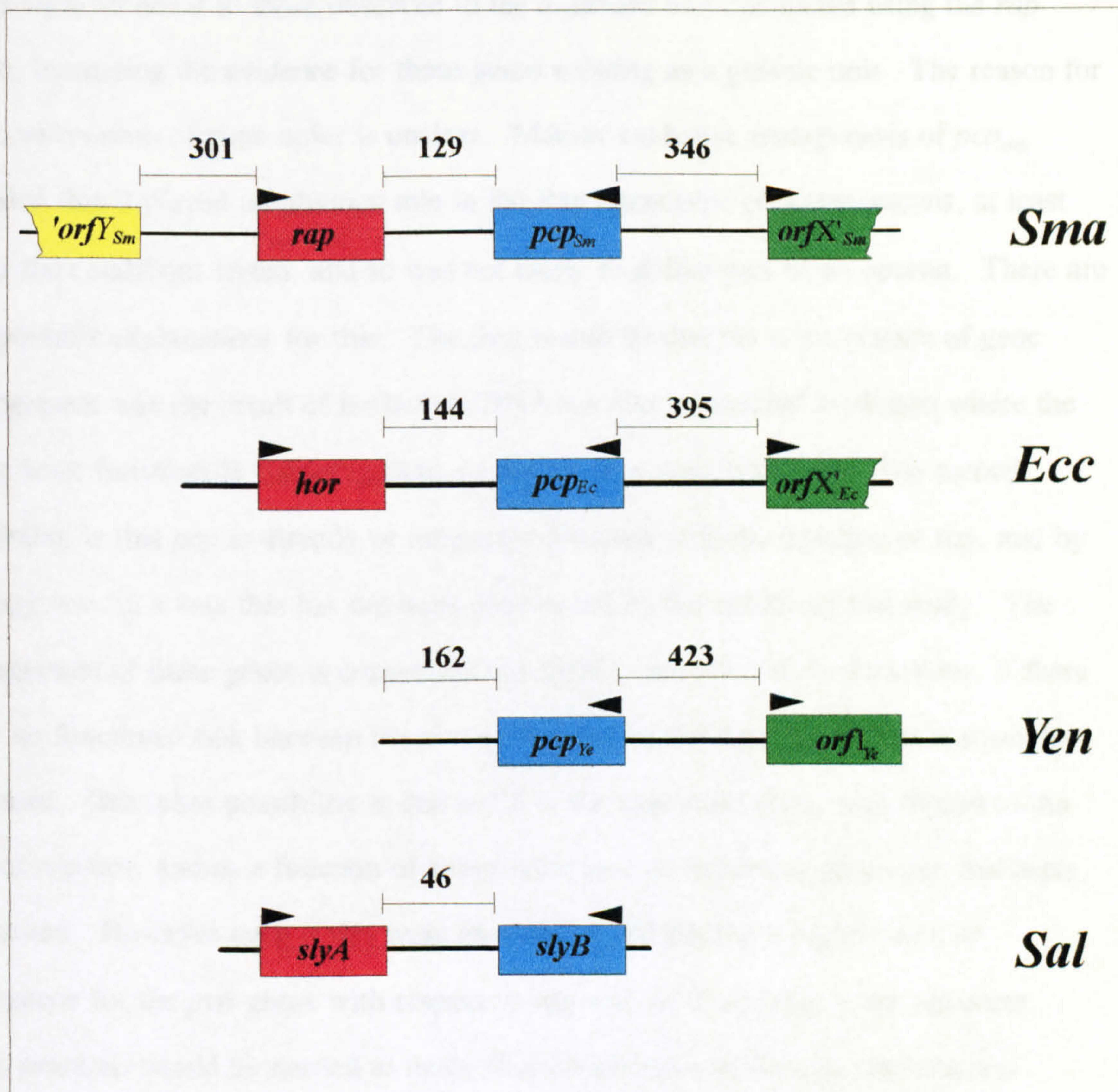


Figure 6.11 Conservation in the gene order of homologues of *pcp*, *rap* and *orfX'*, between *Erwinia*, *Salmonella*, *Serratia* and *Yersinia* (not to scale). The coloured boxes represent open reading frames transcribed in the direction indicated by the arrows. All the size numbers show distance in base pairs. Homologues genes are similarly coloured. *Sma* - *Serratia marcescens* (this study); *Ecc* - *Erwinia carotovora* subspecies *carotovora* (this study); *Yen* - *Yersinia enterocolitica* (Baumler and Hantke, 1992); *Sal* - *Salmonella typhimurium* (Ludwig et al., 1995).

possible *pcp* homologues in the bacterial strains already identified as having *rap* homologues. A filter for a Southern blot, identical to that made in section 5.6, was prepared and the results revealed that *pcp* homologues were represented in all of the *erwinias* tested with one exception, *Ech* SCRI479. The sizes of the *pcp* hybridising bands were identical to those observed in the Southern blot conducted using the *rap* probe, increasing the evidence for these genes existing as a genetic unit. The reason for this conservation of gene order is unclear. Marker exchange mutagenesis of *pcp*_{sm} revealed that it played no obvious role in the Rap phenotype of *S.marcescens*, at least under the conditions tested, and so was not likely to define part of an operon. There are two possible explanations for this. The first would be that the conservation of gene arrangement was the result of horizontal DNA transfer or parallel evolution where the genes were fortuitously conserved and do not act as a functional unit. The second possibility is that *pcp* is directly or indirectly involved with the function of *rap*, and by analogy *hor*, in a way that has not been determined by the results of this study. The arrangement of these genes is considered too highly conserved to be fortuitous, if there were no functional link between the two genes, and so the later possibility is strongly favoured. One other possibility is that *orf'X* is the important gene, with regard to the role of *rap/hor*, and as a function of being between two important genes *pcp* has been conserved. However even if this were true one would predict a higher level of divergence for the *pcp* genes with respect to *rap* and *orf'X* and this is not apparent. More research would be needed to make firm conclusions about this phenomenon.

Other bacteria are already known to have homologues of Pcp: *Enterobacter agglomerans* was shown to have a *pcp* homologue in this study (data not shown), in addition to the published sequence of *pcp* homologues from *Yersinia* and *Haemophilus* (Baumler and Hantke, 1992; Deich *et al.*, 1988). To investigate whether these bacteria, by analogy with *Ecc* and *S.marcescens*, had homologues of *rap* downstream from their respective *pcp* genes, the published sequences of Pcp_{Ye} and Pcp_{Hi} were studied.

6.4 THE SEARCH FOR OTHER HOMOLOGUES OF RAP

6.4.1 INTRODUCTION

The sequence and gene arrangement of the *Serratia* and *Erwinia rap/hor*, *pcp* and *orfX*' genes indicated that there was conservation of these genes as a genetic unit (figure 6.11). Despite the fact that the reasons for this are unclear, it was decided to search the published sequences of the *Yersinia enterocolitica* and *Haemophilus influenzae* for any indication that they also had a *rap* homologue in a similar position downstream of their *pcp* genes.

There was available: 160 bp and 162 bp of published sequence downstream of *Y.enterocolitica* (Baumler & Hantke, [1992]; GenBank accession N°X60448) and *H.influenzae* (Deich, [1988]; GenBank accession N°M18877) *pcp* genes respectively. These sequences were translated in six reading frames using UWGCG TRANSLATE and any possible protein products were aligned with Rap.

The *Yersinia* sequence was predicted to contain the C- terminus of an Orf encoding 12 amino acids (figure 6.12) of a protein which lined up convincingly with Rap, Hor and SlyA (figure 6.13). No such Orf was identified on the *H.influenzae* sequence. This data agreed with that of Southern blots in which no hybridising band was observed for *H.influenzae* (data not shown) but a hybridising band was seen for *Y.enterocolitica* (figure 5.12b).

6.4.2 ISOLATION OF THE *Yersinia* HOMOLOGUE OF *rap*: '*hor_{Ye}*'

A chromosomal library for *Y.enterocolitica* was not available and so the *Yersinia* homologue of *rap* (denoted the *hor_{Ye}* gene) was isolated by polymerase chain reaction (PCR). It was evident from the published sequence that the putative *Y.enterocolitica* *hor_{Ye}* gene and the *pcp_{Ye}* gene were transcribed convergently (figure 6.12) as with the analogous genes in *S.marcescens* and *Ecc*. The primer CyclCyto1 (appendix) had been designed to the highly conserved region upstream of the *rap* and *hor_{Ec}* translational start sites (figure 6.5), if it was assumed that this region was as conserved in *Y.enterocolitica*

then CyclCyto1 could be used as one of the primers to PCR amplify the *hor_{Ye}* gene. A complementary sequencing primer (CyclYen1; appendix) was designed using the published *Y. enterocolitica* sequence (figure 6.12). Because the sequence of the analogous site to which CyclCyto1 was designed to anneal was unknown in *Yersinia*, CyclYen1 was designed to anneal to a site 53 bp from the *HindIII* terminal of the *pcp_{Ye}* published sequence, such that on sequencing any PCR amplified product would, unless it was a spurious PCR product, contain some of this published sequence. The PCR protocol used was as described previously with an annealing temperature of 43°C using *Yersinia enterocolitica* chromosomal DNA as the template.

There were several PCR products amplified, two of which showed up as intense bands on an agarose gel (data not shown). One of these intense bands was 0.5 kb (the predicted size if similar to *rap*) and the other one was approximately 1.5 kb, far bigger than was predicted had CyclCyto1 bound just upstream of the translational start site. This gave rise to two possibilities, either the larger band was spurious PCR product due to miss-priming of Cyclcyto1 or the primer in addition to binding to the predicted position, just upstream of the ATG codon, had bound further upstream of the *hor_{Ye}* gene generating larger PCR products. Because the sequence of the desired product would contain some of the published sequence this was not considered a problem. In fact if CyclCyto1 had bound further upstream it would be an advantage, allowing far more sequence upstream of *hor_{Ye}* to be determined. Both the two major PCR products (0.6 kb and 1.5 kb) were selected for the reasons above and used as templates for cycle sequencing reactions.

The cycle sequencing was carried out using primer CyclYen1 at an initial temperature of 65°C. The temperature for the second round of the protocol was 72°C (section 2.8.8.2). 276 bps of identical sequence was obtained from each of the two PCR products, the sequence derived from both of these PCR products included about 30 bp of the published sequence. Additional primers were designed as the sequence progressed

(shown in figure 6.14) and the sequence obtained from the 0.6 kb and 1.5 kb PCR products has been merged and is shown in figure 6.15.

The *hor_{Y_e}* gene was 432 nucleotides long (position 162-594) and was predicted to encode a protein of 144 amino acids with a calculated molecular weight of 16222 Da and a pI of 7.84 (Figure 6.15). The sequence upstream of *hor_{Y_e}* does not display the same high level of conservation as observed between the *S.marcescens* and *Ecc* nucleotide sequences (figure 6.5). However, it does have one of the partial palindromes detected in these other bacteria as shown in figure 6.15.

The usual ATG initiation codon was not present in the *Yersinia* sequence, the position of the highly conserved ribosome binding site (AGGAGG; figure 6.15) suggested that the first codon of this Orf was the TTG codon. This was an unusual initiation codon, but was consistent with the findings of Ludwig *et al.*, (1995) who reported that SlyA and the SlyA related proteins from *E.coli*, *Shigella* and *Citrobacter* also possessed ribosome binding sites in a position that indicated that the first codon was TTG. This has implications for the efficiency of translation of this Orf. fMet-tRNA_f recognises GUG half as well as AUG and this declines about half again for UUG (Lewin, 1987b). This maybe balanced by the ribosome binding site for *hor_{Y_e}* which matches the consensus exactly (Shine and Dalgarno, 1974).

The *Yersinia* homologue of Rap was very similar to all of the Rap and SlyA homologues identified so far (figure 6.16; table 6.1). It is interesting to note that the amino acid residues defined as important for the function of Rap, by sequencing the *rap* EMS mutants (section 5.3), were also conserved in all of the homologues of Rap and SlyA (figure 6.16), a further indication that these residues are important, whether this is structurally or functionally has not been ascertained by the results of this study.

As previously discussed the homologues of Rap and SlyA share a significant level of homology with a range of bacterial regulatory proteins which fulfil a wide number of roles in a diverse number of bacterial genera. Previous reports have shown that these proteins may constitute a family of small regulatory proteins (based on their

FIGURE 6.14

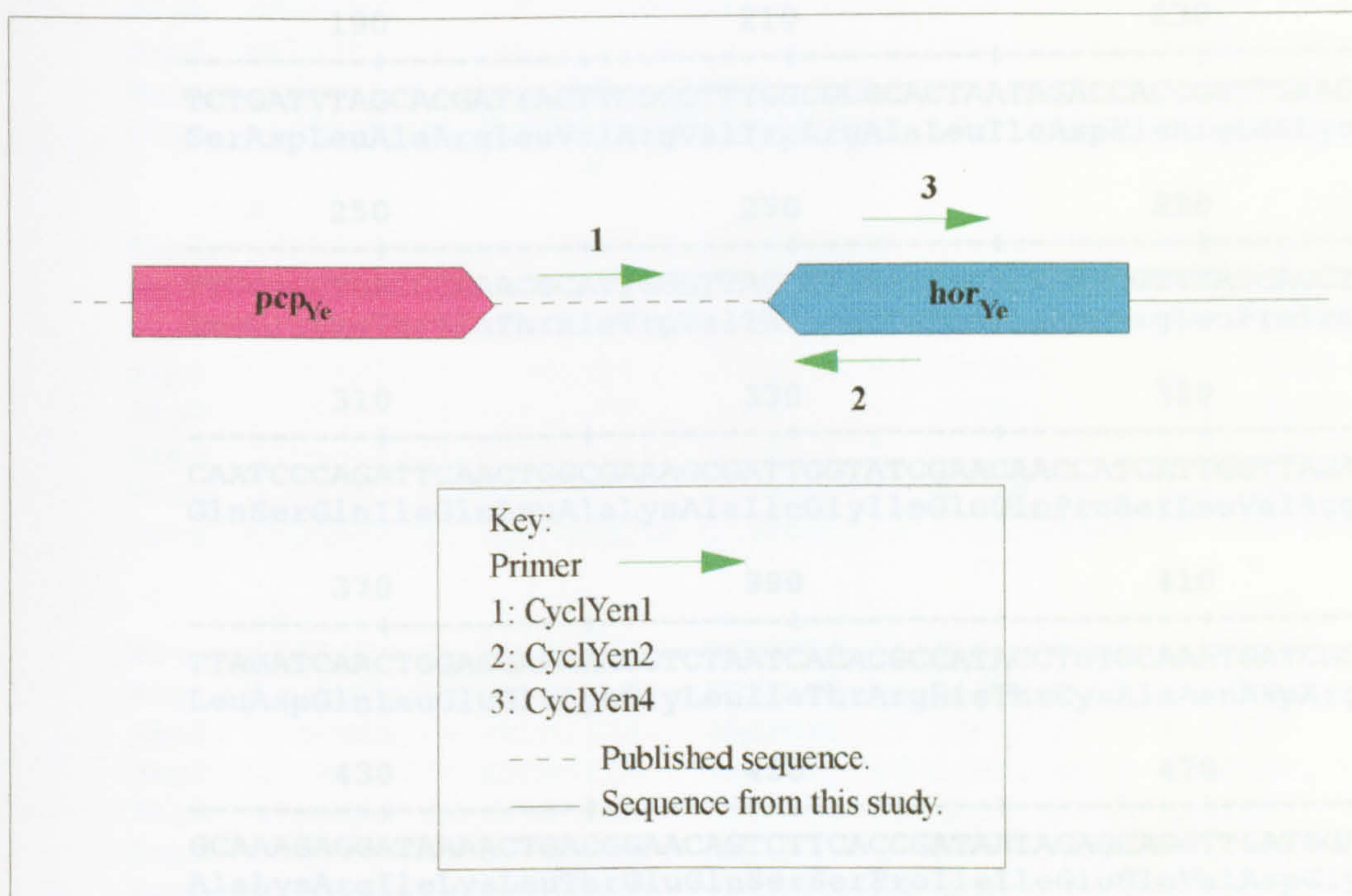


Figure 6.14 A schematic representation of the strategy for sequencing *hor_{Ye}*.

The primers are shown as coloured arrows and the coloured boxes represent the appropriate Orfs. Primer CyclYen1 was designed to the published sequence (indicated by a broken line) from a study by Baumler and Hantke, (1992).

Figure 6.15 Nucleotide sequence (597 bp) upstream of *pcp_{Yc}* (Baumler & Hantke, 1992) encoding the putative *Yersinia* homologue of *rap*. The translation of the sequence is written in blue below the nucleotide sequence. The putative initiation codon was TTG (see text). The termination codon is marked by End in the protein sequence and the ribosome binding site (red; SD-Shine & Dalgarno) is marked in coloured type. The nucleotide sequence between bases 1-330 is only known for one of the DNA strands, however the sequencing of this strand was repeated several times to reduce the likelihood of introducing errors. A palindromic sequence (also found in the *S.marcescens* and *Ecc* sequences figure 6.5) is marked with chevrons.

10 30 50
-----+-----+-----+-----+-----+
TTAGAGTAATTTAGTAAATACAATAATTTTGCTAAAGATTCCCCGTATTTATTGCTATTG

70 90 110
-----+-----+-----+-----+-----+
TCCTAGCTCGACGGTATATATACATTTGTGTTTTTAATGTTAATTACCCAAAATAGCTGG

130 150 180
-----+-----+-----SD-----+---hor_r>>+-----+
CTCTGGTAGTTATGCTAGCACGCTAAATAAAAGGAGGGGCAATTGGAATCGACATTAGGA
 >>>>-<<<< MetGluSerThrLeuGly
 4 4'

190 210 230
-----+-----+-----+-----+-----+
TCTGATTTAGCACGATTAGTTCGCGTTTGGCGCGCACTAATAGACCACCGGTTGAAGCCG
SerAspLeuAlaArgLeuValArgValTrpArgAlaLeuIleAspHisArgLeuLysPro

250 270 290
-----+-----+-----+-----+-----+
TTGGAGCTGACCCAAACGCATTGGGTTACCTTGCATAACATTAATCGTTTACCACCTGAG
LeuGluLeuThrGlnThrHisTrpValThrLeuHisAsnIleAsnArgLeuProProGlu

310 330 350
-----+-----+-----+-----+-----+
CAATCCCAGATTCAACTGGCGAAAGCGATTGGTATCGAACCAACCATCATTGGTTAGAACC
GlnSerGlnIleGlnLeuAlaLysAlaIleGlyIleGluGlnProSerLeuValArgThr

370 390 410
-----+-----+-----+-----+-----+
TTAGATCAACTGGAGGAGAAAGGTCTAATCACACGCCATACCTGTGCAAATGATCGGCGT
LeuAspGlnLeuGluGluLysGlyLeuIleThrArgHisThrCysAlaAsnAspArgArg

430 450 470
-----+-----+-----+-----+-----+
GCAAAGAGGATAAACTGACGGAACAGTCTTCACCGATAATAGAGCAGGTTGATGGAGTC
AlaLysArgIleLysLeuThrGluGlnSerSerProIleIleGluGlnValAspGlyVal

490 510 530
-----+-----+-----+-----+-----+
ATATGTTCCACCCGTAAAGAAATTCTTGGGGGAATTCACCAGATGAAATTGAATTATTA
IleCysSerThrArgLysGluIleLeuGlyGlyIleSerProAspGluIleGluLeuLeu

550 570 590
-----+-----+-----+-----+-----...
TCTGGTTTGATTGACAAGCTTGAGAGAAACATTATTCAACTACAAAGTAAGTAAAAT...
SerGlyLeuIleAspLysLeuGluArgAsnIleIleGlnLeuGlnSerLysEnd

FIGURE 6.16

	1				50
Hor _{Ec}	..MELPLGSD	LARLVRVWRA	LVDHRLKPLE	LTQTHWVTLH	NIYHLPPGQS
Rap	..MELPLGSD	LARLVRVWRA	LIDHRLKPLE	LTQTHWVTLH	NIHELPPGQS
Srp1	MKLESPLGSD	LARLVRIWRA	LIDHRLKPLE	LTQTHWVTLH	NIHQLPPDQS
Srp2	MKLESPLGSD	LARLVRIWRA	LIDHRLKPLE	LTQTHWVTLH	NIHQLPPDQS
SlyA	MKLESPLGSD	LARLVRIWRA	LIDHRLKPLE	LTQTHWVTLH	NIHQLPPDQS
Srp3	MKLESPLGSD	LARLVRIWRA	LIDHRLKPLE	LTQTHWVTLH	NIHQLPPDQS
Hor _{Yc}	MESTLGSD	LARLVRVWRA	LIDHRLKPLE	LTQTHWVTLH	NINRLPPEQS
Cons	--?E-PLGSD	LARLVRIWRA	LIDHRLKPLE	LTQTHWVTLH	NI--LPP-QS
		*			
	51				100
Hor _{Ec}	QIQLAKAIGI	EQPSLVRTL	QLEEKGLITR	HVCAHDRRAK	RIMLTESAEP
Rap	QIQLAKAIGI	EQPSLVRTL	QLEDKGLITR	HICVHDRRAK	RIMLTDMADP
Srp1	QIQLAKAIGI	EQPSLVRTL	QLEEKGLISR	QTCASDRRAK	RIKLTEKAEP
Srp2	QIQLAKAIGI	EQPSLVRTL	QLEEKGLISR	QTCASDRRAK	RIKLTEKAEP
SlyA	QIQLAKAIGI	EQPSLVRTL	QLEDKGLISR	QTCASDRRAK	RIKLTEKAEP
Srp3	QIQLAKAIGI	EQPSLVRTVD	QLEDKGLISR	QTCATDRRAK	AIKLTEKAEP
Hor _{Yc}	QIQLAKAIGI	EQPSLVRTL	QLEEKGLITR	HTCANDRRRAK	RIKLTEQSSP
Cons	QIQLAKAIGI	EQPSLVRTL	QLEEKGLI-R	--CA-DRRAK	RI-LTE-AEP
		*		*	*
	101				147
Hor _{Ec}	IIQAVNGVIS	HTRSEVLFGI	TPEQVDELAL	LVSRLKKNIL	ALHENQA*
Rap	IIQAVNDVID	QTRSEILNGI	TPEEVSELAT	IISRLESNIL	SLYEMQS*
Srp1	LISEMEAVIN	KTRAEILHGI	SAEELEQLIT	LIAKLEHNII	ELHSHD*
Srp2	LISEMEAVIN	KTRAEILHGI	SAEELEQLIT	LIAKLEHNII	ELHSHD*.
SlyA	LIAEMEEVIH	KTRGEILAGI	SSEEIELLIK	LIAKLEHNIM	ELHSHD*
Srp3	LITEMEAVTN	KPRGEILEGI	PA.....
Hor _{Yc}	IIEQVDGVIC	STRKEILGGI	SPDEIELLSG	LIDKLERNII	QLQSK*..
Cons	-I-----VI-	-TR-EIL-GI	--E-----L--	-I--LE-NI-	-L-----

Figure 6.16 A multiple line up of all the known closely related homologues of SlyA and Rap (Serratia marcescens; regulation of antibiotic and pigment production; this study), Hor_{Ec} (Erwinia carotovora subsp carotovora; this study), SlyA (Salmonella typhimurium; Salmolysin; Libby et al., 1994; Accession number UO3842 GenBank) and SlyA related proteins (Srp), for which there were no published names or accession numbers. For the purposes of this study they were nominally designated as Srp1-3 (Srp1- Escherichia coli; Srp2-Shigella flexineri and Srp3- Citrobacter diversus; Ludwig et al., 1995). The consensus is marked in blue showing the positions with at least 6 identical or conserved amino acids with amino acids totally conserved highlighted in red. Residues defined as important by sequencing the EMS generated S.marcescens rap mutants (section 5.3) indicated with an asterisk. The ‘?’ in the consensus indicates the position proposed to be the first methionine residue in all the proteins shown.

homology; Del Castillo *et al.*, 1991; Marklund *et al.*, 1992; Dehoux and Cossart, 1995). Figure 6.17 describes the relatedness of these proteins and the homologues of Rap, discovered in this study, as a phylogenetic dendrogram. From the dendrogram it is easy to see that Rap, Hor_{Ec}, Hor_{Ye} and the SlyA related proteins constitute a distinct subgroup of this proposed wider family of bacterial regulatory proteins. Within this subfamily Hor_{Ye} holds a central position between the SlyA related proteins and Rap and Hor_{Ec}. The significance of this is not clear and if more homologues of *rap* are sequenced this may be shown to be artifactual.

TABLE 6.1

	Rap	Hor _{Ec}	Hor _{Ye}	SlyA	Srp1	Srp2	Srp3
Rap	*	-	-	-	-	-	-
Hor _{Ec}	83	*	-	-	-	-	-
Hor _{Ye}	74	73	*	-	-	-	-
SlyA	71.5	71	78	*	-	-	-
Srp1	71.5	71	78	81	*	-	-
Srp2	71.5	71	78	81	100	*	-
Srp3	75	74	77	81	91	91	*

Table 6.1 A comparison of all the homologous Rap and SlyA related proteins (Srp) from this study, Libby *et al.*, (1994) and Ludwig *et al.*, (1995). Figures represent the % amino acid identity and were calculated using UWGCG BESTFIT program. Rap, Hor_{Ec} and Hor_{Ye} this study; Srp1- *Escherichia coli*; Srp2-*Shigella flexineri* and Srp3- *Citrobacter diversus*; Ludwig *et al.*, (1995).

6.5 DISCUSSION

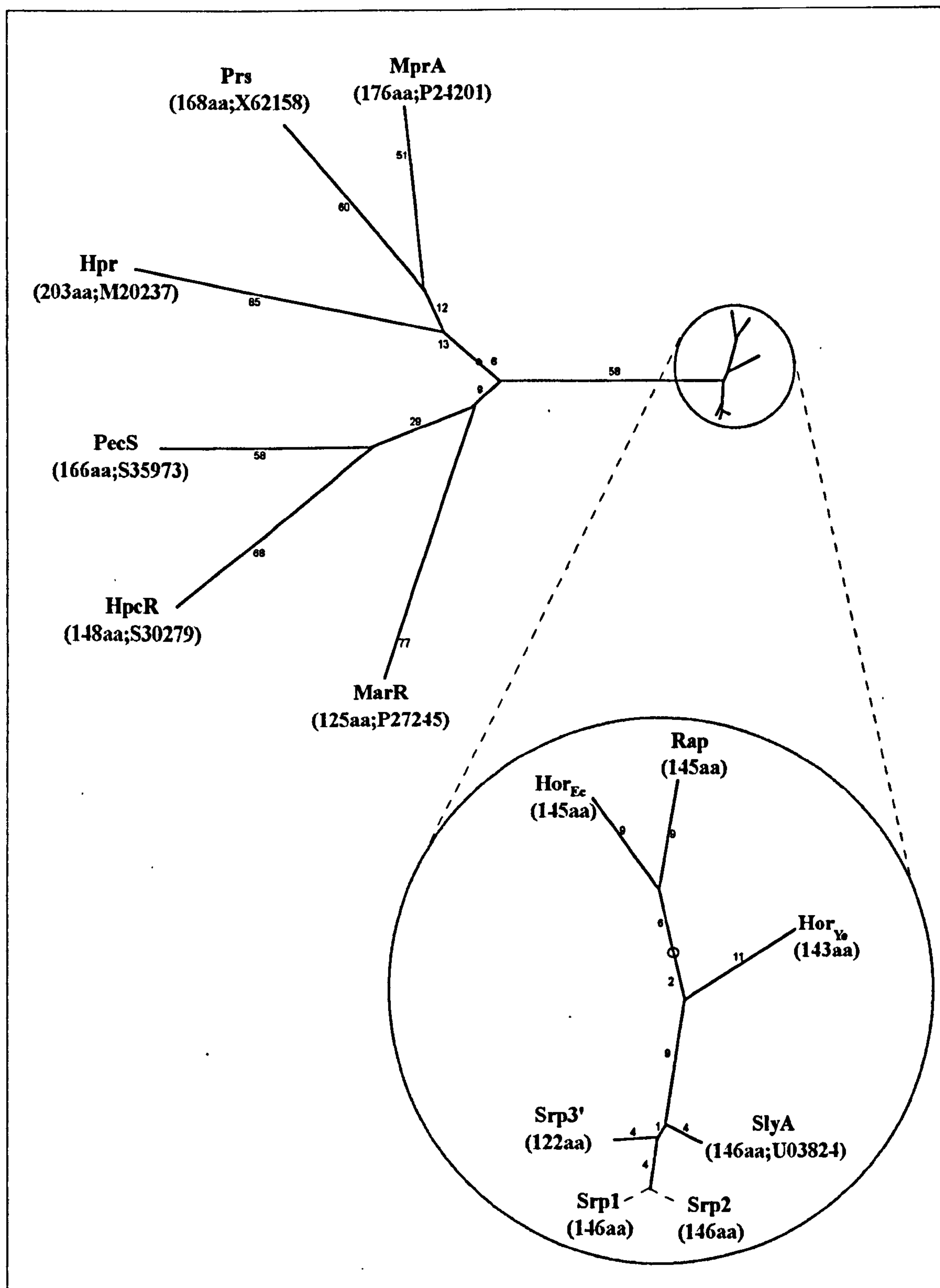
The conservation of the *orfX'*_{Ec}, *pcp*_{Ec} and *hor*_{Ec} gene arrangement was also observed in *Y.enterocolitica*, with the *Yersinia* homologue of *rap* being located 121 bp upstream of *pcp*_{Ye}. This finding contradicts the findings of both Libby *et al.*, (1994) and the more recent findings of Ludwig *et al.* ., (1995) in which *Yersinia* was concluded to be lacking such a gene.

Hor_{Ye} aligned well with the other Rap homologues adding to a growing family of highly related small proteins. It is very clear from the dendrogram that the homologues

Figure 6.17 A dendrogram of the wider family of Rap homologues.

The dendrogram includes: Rap, Hor_{Ec} and Hor_{Y₆} (this study); MprA (Del Castillo *et al.*, 1991; MarR (Cohen *et al.*, 1993; Ariza *et al.*, 1994); PecS (Reverchon *et al.*, 1994); SlyA (Libby *et al.*, 1994); SlyA related proteins- Srp1-3 (Ludwig *et al.*, 1995); HpcR (Roper *et al.*, 1993); Prs (17 KDa protein; Marklund *et al.*, 1992); PetP (Tokito and Daldal, 1992) and Hpr (Perego and Hoch, 1988). The dendrogram was drawn using ALLALL (section 2.9; based on the Needleman-Wunsch algorithm) which measures evolutionary distance in PAM units (the number of point accepted mutations per 100 residues separating two sequences, taking into account residues with similar properties). The statistical variance of the PAM units between aligned sequences is expressed as a probability of relatedness, the larger the PAM distance the less related are the proteins as shown above (see Gonnet *et al.*, 1992). Figures contained in the brackets indicate the length of the protein (aa- amino acids) and the accession number where available

FIGURE 6.17



of Rap and SlyA form a subgroup of what appears to be a family of more distantly related proteins, identified in diverse backgrounds and fulfilling a multitude of different roles. Perhaps this is a function of this subgroup of proteins fulfilling very similar roles in their respective hosts, in contrast to the multiple roles performed by the wider family of proteins. It is interesting to note from the dendrogram that although PecS and Hor_{Ec} are both found in *Erwinia* species, Hor_{Ec} shares a far higher identity to a protein from *Yersinia* than to PecS. This may indicate that these two proteins are distinct and are probably not functional analogues of each other. In considering this proposed wider family of proteins, some benefit may be drawn from discussing what is known about their mode of action, with regard to the possible functioning of proteins discovered in this study:

The genes *pecS* and *pecM* were discovered in *Erwinia chrysanthemi* (Reverchon *et al.*, 1994). Deletion of either one of these genes led to the over expression of the exoenzymes pectate lyase and cellulase as well as the induction of a cryptic blue pigment. PecS is a protein of 166 amino acids in length which has no obvious DNA binding motifs and yet has been shown to bind DNA (Praillet *et al.*, 1996) thereby repressing the production of exoenzymes and pigment.

MarR is another homologue on which much research has been focused. This protein is a repressor of chromosomal mediated multiple antibiotic resistance (MAR) in *E.coli*, which is activated by the *marROAB* operon (Cohen *et al.*, 1993a). Constitutive MAR mutants were isolated, which had a heightened resistance to a number of structurally unrelated compounds. All of these mutants were found to have a mutation in either *marR* or in *marO* which is the operator region where MarR has been shown to bind, situated just upstream of the *mar* operon (Martin and Rosner, 1995). Inducers of MAR have been found to affect MarR directly; salicylate prevents the binding of MarR to the operator region thereby de-repressing the *marORAB* operon (Martin and Rosner, 1995; Cohen *et al.*, 1993b).

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MarA was found to be the positive activator of the MAR phenotype and is related to the AraC family of transcriptional activators (Cohen *et al.*, 1993a). MarA is responsible for the activation of many unlinked genes (Seoane and Levy, 1995) which are involved in roles ranging from resistance to antibiotics, weak aromatic acids (such as salicylate) and phage, to genes involved in resistance to oxidative stress (Greenberg *et al.*, 1991; Ariza *et al.*, 1994).

Other homologues for which the function of the protein was not quite so well characterised included PetP, MprA, HpcR, Hpr and an un-named gene encoding a 17kDa protein from uropathogenic *E.coli*. PetP is a protein discovered in *Rhodobacter capsulatus* (166 amino acids; Tokito and Daldal, 1992); the deletion of this gene severely affected growth. MprA (176 amino acids) controls the synthesis of microcins B17 and C7. A chromosomal genetic lesion in *mprA* caused up to five fold induction of the transcription of *mcb-lacZ* fusions, classifying MprA as a repressor of the *mcbABCDEF* operon (Del Castillo *et al.*, 1991).

HpcR (148 amino acids) is a protein from *E.coli*, discovered whilst conducting research into the degradation of aromatic compounds. Whilst the exact mode of action of this protein is not known, it has been shown to be a repressor of the homoprotocatechuate degradative operon (Roper *et al.*, 1993). Hpr is a negative regulator of sporulation and protease production in *Bacillus subtilis* (Perego and Hoch, 1988), with a 16-37 fold increase in protease production in a *hpr* mutant.

Lastly a gene encoding a 17 kDa cytoplasmic protein in uropathogenic *E.coli* which is located in the *prs* cluster that encodes the synthesis and assembly of Pap pili: The ability to bind to the host is a major virulence determinant for *E.coli* causing upper urinary tract infections. This binding is achieved by the presence of G-adhesins located at the tips of the Pap pili. The 17 kDa protein gene is located at the end of the *prs* cluster and is highly conserved between *E.coli* strains, however the relevance of this gene, in terms of regulation of the operon, remains to be established (Marklund *et al.*, 1992).

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In summary, the homologues of *rap* identified and sequenced from *Serratia marcescens*, *Erwinia carotovora* subspecies *carotovora* and *Yersinia enterocolitica*, are members of a growing family of highly conserved proteins. This family has more distantly related homologues which are implicated in virulence of plant and human pathogens, multiple drug resistance and catabolism of xenobiotic compounds.

The most obvious similarity between these proteins is their small size ranging from 125 amino acids for MarR to 203 amino acids for Hpr and the fact that they have a global effect on a number of phenotypes. These proteins do not have obvious DNA binding motifs and yet some of these homologues have been shown to bind DNA. MarR has been shown not only to bind DNA, but also act as a sensor protein to which an inducer binds thereby derepressing the *marRAB* operon.

It was not clear what function the closely related Rap homologues (Hor_{Ec}, SlyA, and Hor_{Ye}) performed or if they bound DNA. The function of SlyA in its cognate host was not well understood and so it was important to define the function of the *hor_{Ec}* gene in the genetically amenable plant pathogen *Erwinia carotovora* subspecies *carotovora*. Unfortunately there was not enough time left in my PhD to progress any further with the study of the *Yersinia rap* homologue, however the function of *hor_{Ec}* in *Ecc* was studied and is discussed in the next chapter.

CHAPTER 12

In summary, the homologues of *rap* identified and sequenced from *Serratia marcescens*, *Erwinia carotovora* subspecies *carotovora* and *Yersinia enterocolitica*, are members of a growing family of highly conserved proteins. This family has more distantly related homologues which are implicated in virulence of plant and human pathogens, multiple drug resistance and catabolism of xenobiotic compounds.

The most obvious similarity between these proteins is their small size ranging from 125 amino acids for MarR to 203 amino acids for Hpr and the fact that they have a global effect on a number of phenotypes. These proteins do not have obvious DNA binding motifs and yet some of these homologues have been shown to bind DNA. MarR has been shown not only to bind DNA, but also act as a sensor protein to which an inducer binds thereby derepressing the *marRAB* operon.

It was not clear what function the closely related Rap homologues (Hor_{Ec}, SlyA, and Hor_{Ye}) performed or if they bound DNA. The function of SlyA in its cognate host was not well understood and so it was important to define the function of the *hor_{Ec}* gene in the genetically amenable plant pathogen *Erwinia carotovora* subspecies *carotovora*. Unfortunately there was not enough time left in my PhD to progress any further with the study of the *Yersinia rap* homologue, however the function of *hor_{Ec}* in *Ecc* was studied and is discussed in the next chapter.

***INACTIVATION OF THE Ecc hor
GENE BY ALLELIC EXCHANGE***

7.0 PREFACE

As this study progressed it became clear that Rap, Hor_{Ec}, Hor_{Ye} and SlyA (Libby *et al.*, 1994) belonged to a wider family of small protein regulators controlling multiple and diverse phenotypes in various bacteria: antibiotic, pigment production, exoenzyme production and virulence factors in plant and animal pathogens as well as multiple antibiotic resistance and catabolism of xenobiotic compounds in *E.coli*. The two *hor* genes from *Erwinia carotovora* subspecies *carotovora* (*Ecc*) and *Yersinia enterocolitica* had been cloned and sequenced (chapter 6) using DNA probes designed from the *S.marcescens rap* gene. No function had yet been associated with these genes. In order to investigate the phenotype of a Hor_{Ec} mutant in *Erwinia*, allelic exchange was used to create a selectable chromosomally located *hor::kan^R* mutation (*kan^R*- kanamycin resistance gene from pACYC177 derived from Tn903; Chang and Cohen, 1978). This selectable *hor::kan^R* allele was then used in later experiments for strain construction: laboratory *Ecc* strains which had *lacZ* promoter fusions to various genes were transduced using phage KP (Toth, 1991; Toth *et al.*, 1993) propagated on the kanamycin resistant *hor^r* mutant. Unfortunately there was insufficient time to further investigate the *Yersinia* homologue of *rap* - *hor_{Ye}*, but this will be the subject of a new project.

7.1 ALLELIC EXCHANGE OF *hor_{Ec}*

The strategy and the molecular events leading to the construction of a chromosomal located *hor::kan^R* allele were similar to those used in the marker exchange of *rap* and *pcp_{Sm}* (section 5.4) and so will only briefly be detailed in this section.

7.1.1 CONSTRUCTION OF THE *hor* MARKER EXCHANGE PLASMID pKNG25H

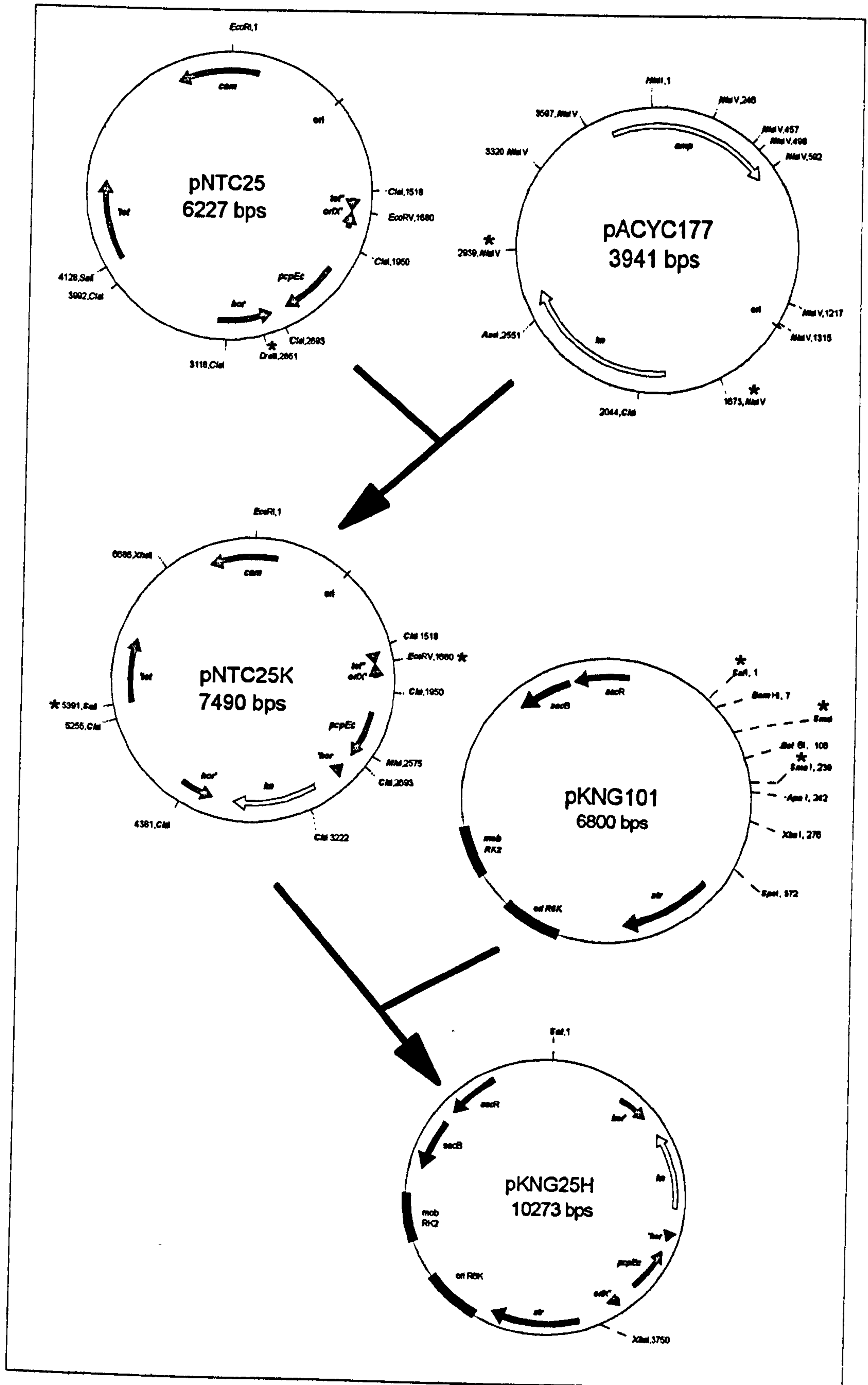
The 1266 bp *Nla*IV restriction fragment containing the kanamycin resistance gene was excised from plasmid pACYC177 (Chang and Cohen, 1978) and cloned into the unique *Dra*III site of pNTC25 (section 6.1.3), thereby disrupting the *hor* gene (figure 7.1). This mutant allele was predicted to encode the synthesis of a truncated

Legend to Figure 7.1

The strategy for the construction of the *hor* marker exchange plasmid pKNG25H.

The 1266 bp kanamycin resistance cassette was excised from pACYC177 (Chang and Cohen, 1978) using *Nla*IV and ligated into the unique *Dra*III site of pNTC25, transformants were selected on NBA supplemented with chloramphenicol and kanamycin. The 3714 bp *Eco*RV-*Sal*I fragment containing the kanamycin cassette was cut from pNTC25K and ligated into pKNG101 cut with the compatible enzymes *Sal*I and *Sma*I. This ligation mix was used to electroporate *E.coli* strain CC118 (λ *pir*) and transformants were selected on NBA supplemented with streptomycin and kanamycin. Plasmids named pKNG25H was purified from one of these transformants and used for marker exchange (see text). Genes are colour coded corresponding to the parental plasmid. Asterisks mark important sites used during the construction of the various plasmids. The published restriction map of pKNG101 shows a unique *Sma*I site. However this is not the case and there is a second site between the published *Sma*I site and the *Sal*I site (S.McGowan.pers.comm). This necessitated the use of *Sal*I for digestion before *Sma*I because *Sal*I cannot cut efficiently close to the end of a DNA fragment. Plasmid resistance gene abbreviations: *kn*- kanamycin resistance; *str*- streptomycin resistance; *tet*- tetracycline resistance; *amp*- ampicillin resistance and *cam* chloramphenicol resistance.

FIGURE 7.1



protein missing the C-terminal 36 amino acids of the wild type protein (the wild type Hor protein -146 amino acids). The mutant *hor::kan^R* allele was then cloned into pKNG101 forming pKNG25H as described in figure 7.1. A summary of the events during the integration and resolution of pKNG25H is depicted in figure 7.2.

7.1.2 SOUTHERN BLOT OF *hor* MARKER EXCHANGE MUTANTS

Chromosomal DNA, prepared from four colonies (named ATM101-4) recovered after marker exchange, were digested with restriction enzymes *Mlu*I and *Sal*I. The digested chromosomal DNA was run on an agarose gel overnight and prepared for a Southern blot (figures 7.3a and b). These two enzymes do not cut within the kanamycin cassette (figure 7.2) and so marker exchange mutants would exhibit a band shift of 1266 bp (equal to the size of the kanamycin resistance cassette). The probe used in this Southern blot was a PCR generated, DIG labelled, probe (597 bp) encoding the *hor* Orf. The primers used to make this probe were Cyclcyto1 and Cyclcyto2 (appendix) with a PCR annealing temperature of 44°C. All other parameters were as previously described (section 2.6).

The Southern blot (figure 7.3) clearly showed a band shift of approximately 1266 bp for the marker exchanged strains. The wild type control lane presented a band of ~1500 bp and the marker exchange mutants exhibited a band of ~2760 bp, confirming that the marker exchange had been successful. The next priority for this study was to identify the phenotype of these *hor*⁻ mutants.

7.2 PRELIMINARY ANALYSIS OF THE HOR PHENOTYPE

Initial investigation into the possible phenotypic effects of the *hor* gene mutation were directed towards looking at exoenzyme and antibiotic production. The rationale for this was that the *Serratia rap* mutants were carbapenem negative (Car⁻; section 3.0) and related protein regulators such as PecS (Reverchon *et al.*, 1994) and Hpr (Perego and Hoch, 1988) have been shown to be involved in exoenzyme production. The initial

1. The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that proper record-keeping is essential for the integrity of the financial system and for the ability to detect and prevent fraud.

2. The second part of the document outlines the specific procedures for recording transactions. It details the steps involved in the accounting cycle, from identifying the transaction to posting it to the appropriate ledger account.

3. The third part of the document discusses the importance of reconciling the books. It explains how regular reconciliations help to ensure that the books are balanced and that there are no discrepancies between the recorded transactions and the actual transactions.

4. The fourth part of the document discusses the importance of auditing the books. It explains how audits help to detect errors and fraud, and to ensure that the financial statements are accurate and reliable.

5. The fifth part of the document discusses the importance of maintaining the books for a long period of time. It explains that books should be kept for at least seven years, and that they should be stored in a secure and accessible location.

Legend to Figure 7.2

A summary of the events leading to the marker exchange of *hor*

Plasmid pKNG25H was integrated into the chromosome by homologous recombination (A), generating a strain that was kanamycin resistant, streptomycin resistant, sensitive to high concentrations of sucrose and temporarily merodiploid with respect to *hor* (B). The integrated pKNG25H plasmid in the *Ecc* chromosome was flanked by regions of homology. By selecting on high sucrose media (10% sucrose) containing kanamycin (5 µg/ml) the second recombination event was forced (C; see section 5.4.1) leaving a chromosomally located *hor::kan^R* allele (D). The excised plasmid is lost because the *Ecc* host does not have the λ *pir* gene required for its maintenance. (antibiotic resistance gene abbreviations: *str*- streptomycin resistance; *kan^R*- kanamycin resistance)

FIGURE 7.2

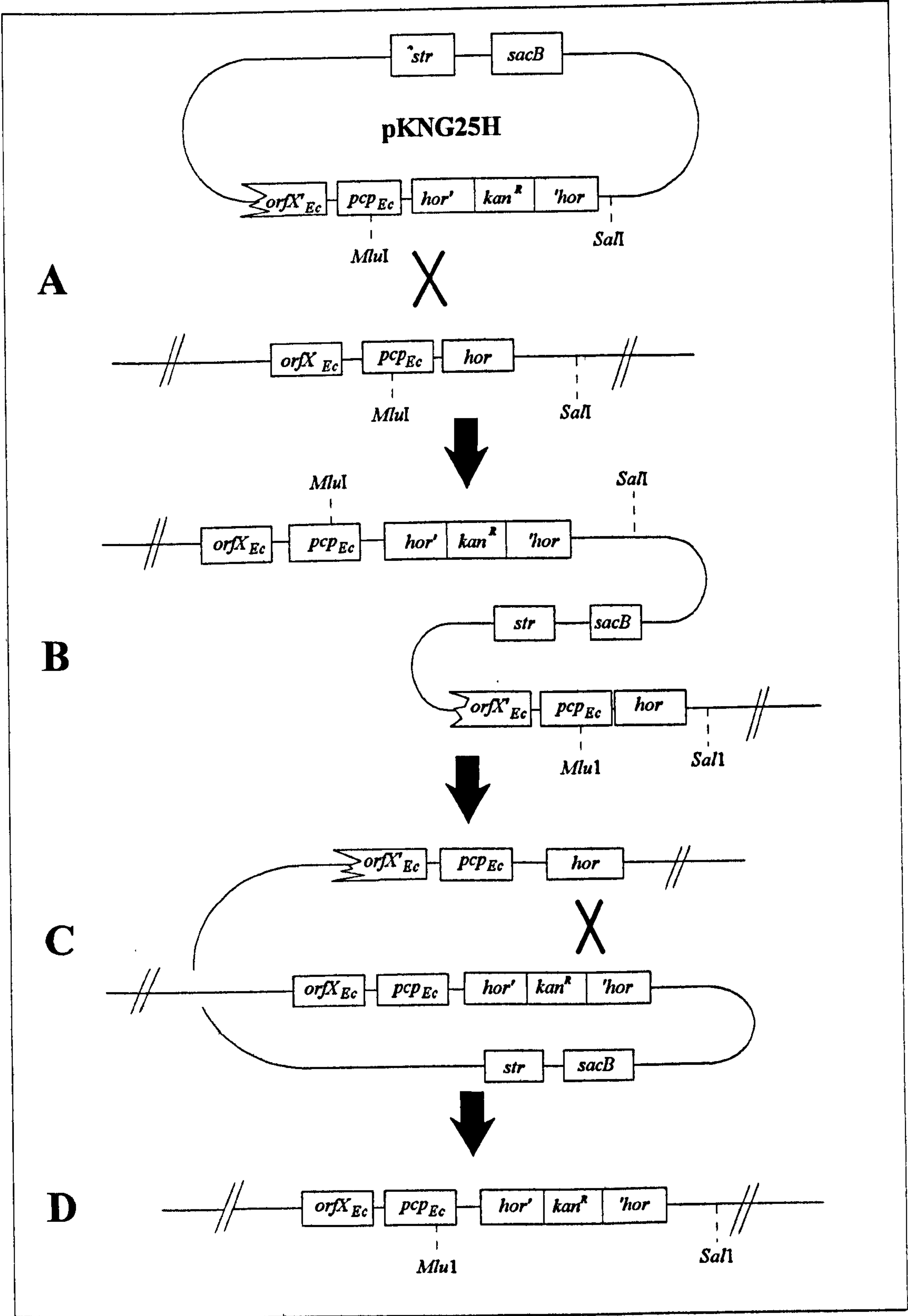


FIGURE 7.3a

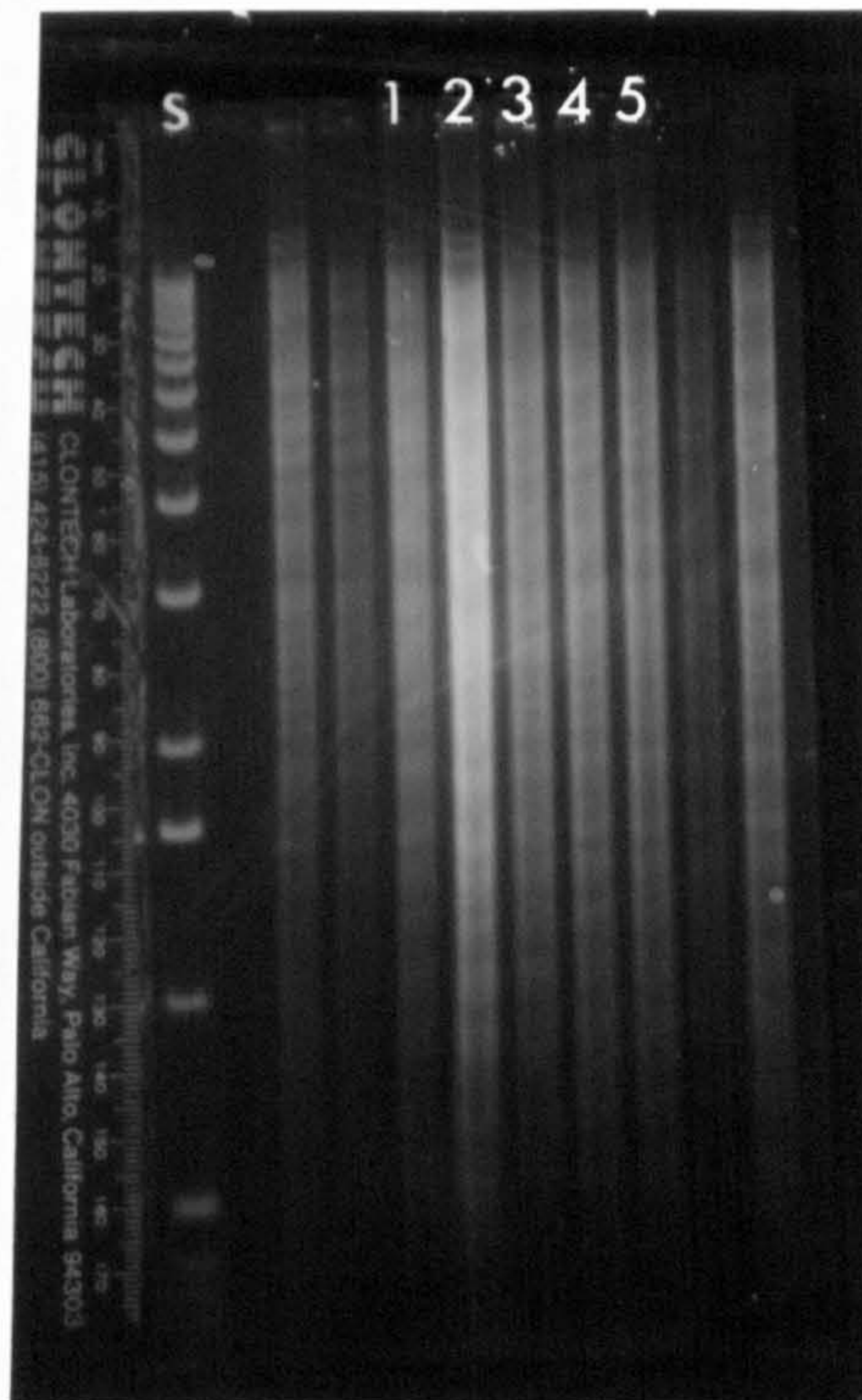


Figure 7.3a Restriction enzyme digests of the putative, *Ecc hor* marker exchange mutants
Chromosomal DNA of the putative *hor* marker exchange mutants cut with restriction enzymes *Mlu*I - *Sal*I

Key to figures 7.3a and 7.3b				
Chromosomal DNA from:-				
Lane: S	1 kb ladder			
1	Wild type <i>Ecc</i> strain	ATTn10		
2	Marker exchange mutant	ATM101		
3	" " " "	ATM102		
4	" " " "	ATM103		
5	" " " "	ATM104		

FIGURE 7.3b

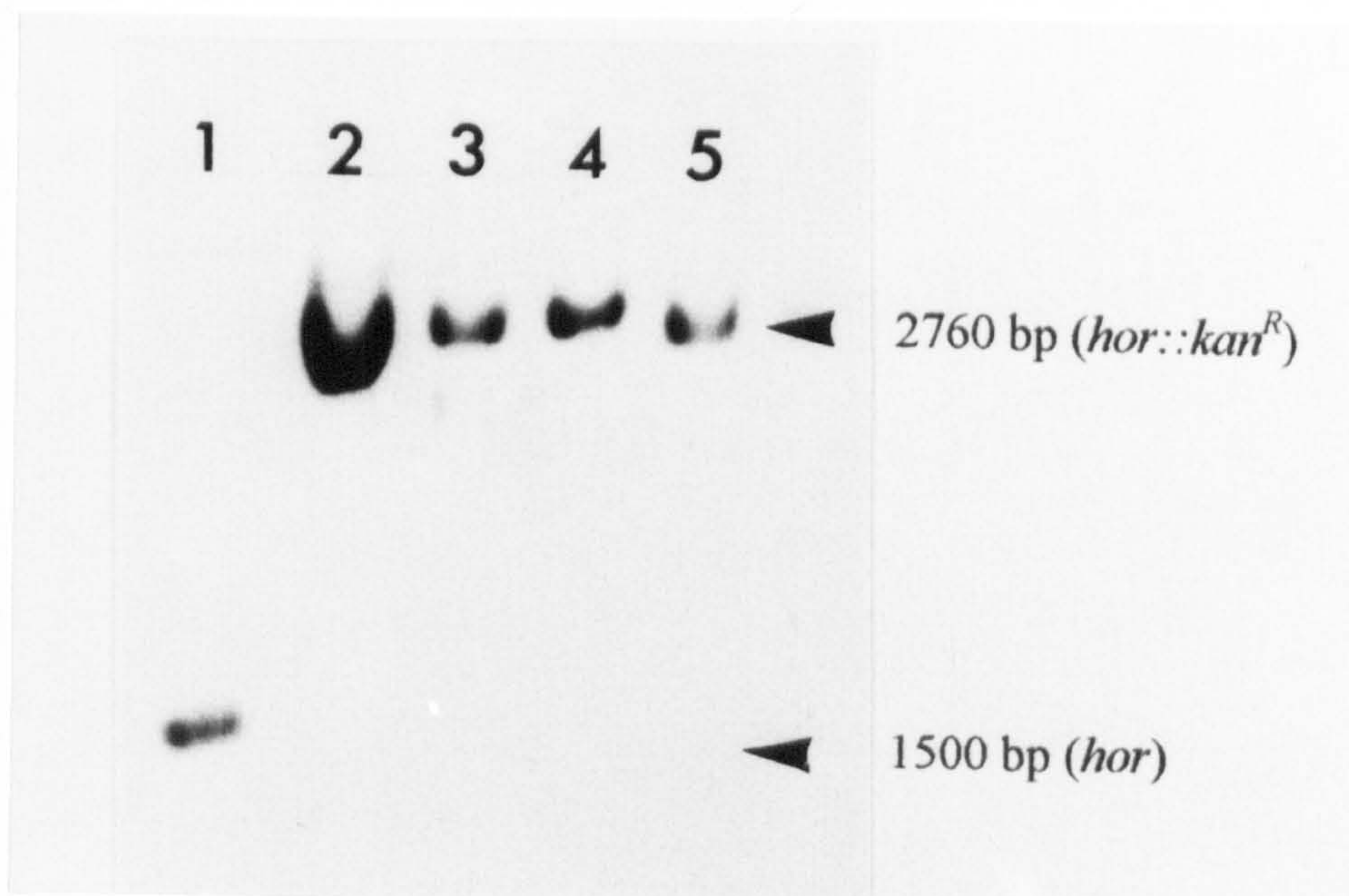


Figure 7.3b Southern blot of putative *hor* mutants recovered after marker exchange.

The filter was probed with a 597 bp *hor* DIG probe made by PCR. The hybridisation temperature for this blot was 55°C. The *Ecc* strain ATTn10 exhibited a hybridising band of ~1500 bp (lane 1). All the 'marker exchanged' strains (see key) had hybridising bands of ~2760 bp, equal to the *Mlu*I- *Sal*I band containing the *hor::kan^R* allele (see text).

tests took the form of plate assays for exoenzyme production and the *E.coli* strain ESS plate bioassay for carbapenem production as shown in figure 7.4.

The results from the plate assays for exoenzymes and carbapenem production were very unexpected. The marker exchange mutants lacked any visible halo on the *E.coli* strain ESS bioassay plate, indicating a total cessation in the production of carbapenem in the *hor* mutants. In addition there was a significant reduction in the halo size on the pectate lyase assay plate (Pel). Less obvious were the reduced halo sizes observed on the cellulase (Cel) and protease (Prt) assay plates. These tests were repeated several times and the exoenzyme and antibiotic phenotype was found to be reproducible.

The putative *Ecc* carbapenem operon is thought to consists of nine genes which are proposed to direct the synthesis of antibiotic in a multistep pathway (McGowan in preparation). Because a bioassay was used to detect carbapenem production, it was impossible to determine whether the Hor protein regulated the pathway as a whole or just part of it. If the Hor protein only regulated part of the putative carbapenem biosynthetic pathway then a non-antimicrobial precursor such as carbapenam could have been produced and for obvious reasons not detected on the bioassay. To address this the *hor* mutants were sent to our collaborating group in Nottingham for testing. HPLC analysis indicated that these mutants produced no detectable carbapenem or any of its known precursors, as defined within the limits of HPLC (P.Williams, pers.comm).

7.2.1 COMPLEMENTATION ANALYSIS OF THE *Ecc hor* MARKER EXCHANGE MUTANTS

To investigate whether the *hor* gene could complement the marker exchange mutants *in trans*, plasmid pNTC25 (section 6.1.3) was introduced by electroporation into *Ecc* strain ATM101 and transformants were selected on NBA supplemented with chloramphenicol. Selected colonies were tested on exoenzyme assay plates for pectate lyase, cellulase and protease production using indicator plate assays (section 2.14), in addition to the *E.coli* strain ESS bioassay for carbapenem production (section 2.13).

FIGURE 7.4

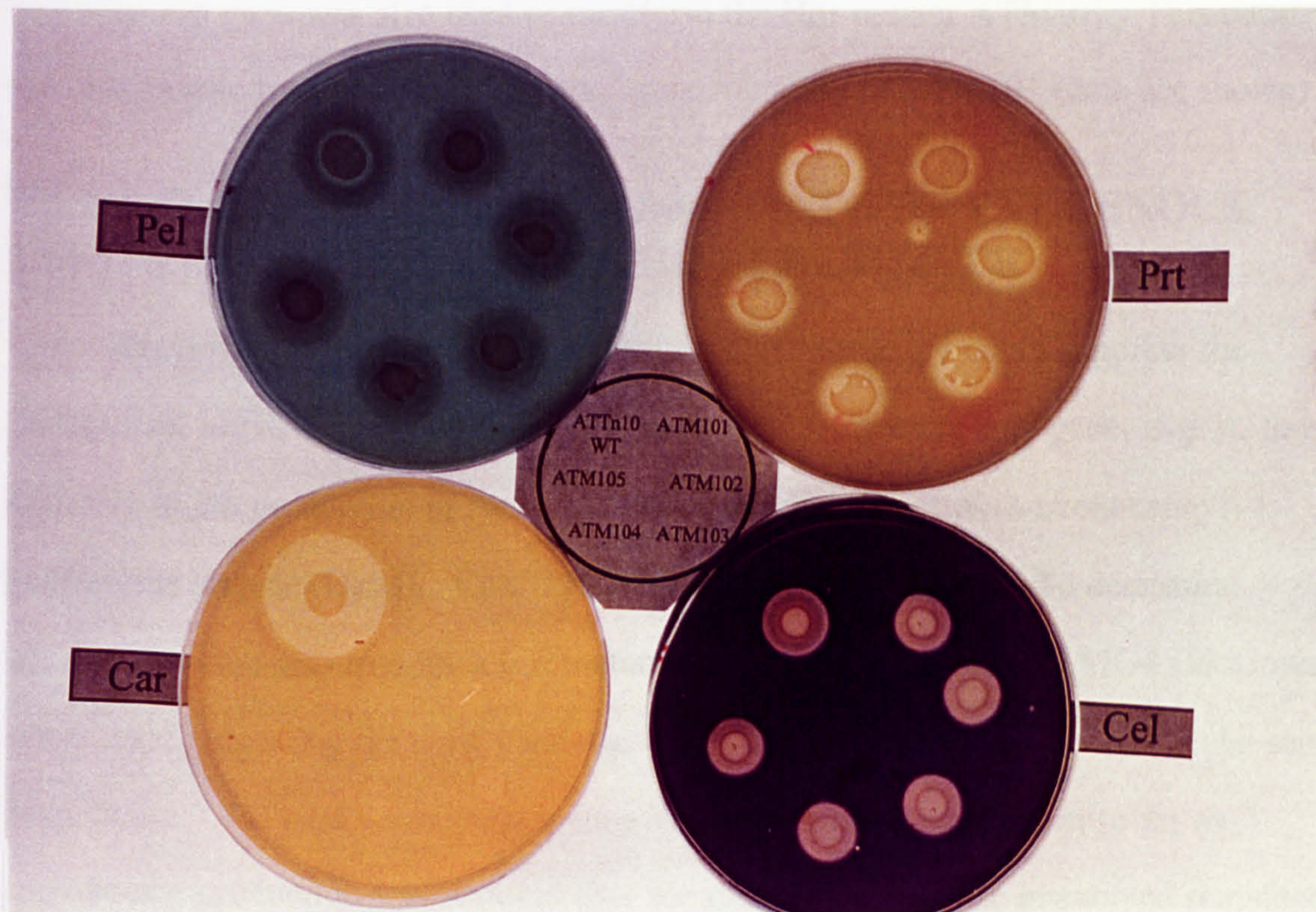


Figure 7.4 Phenotypic analysis of the *Ecc hor::kan^R* marker exchange mutants

Plate assays for exoenzyme and antibiotic production of five *Ecc hor::kan^R* marker exchange strains ATM101-105 with the Wild type (WT) strain ATTh10 strain as control. The exoenzyme plate assays were Pel - pectate lyase; Prt - protease; Cel - cellulase; and Car the *E.coli* strain ESS bioassay for carbapenem production. A halo or clear zone of antibiosis indicates exoenzyme or antibiotic production respectively.

ATM101 transformants containing pNTC25 continued to exhibit the Hor phenotype and did not demonstrate even partial complementation (data not shown). This had not been the case for the complementation of the *S.marcescens rap* mutants where carbapenem and pigment production was restored by the *rap* and *hor* genes *in trans* (section 3.0: data not shown). Following on from this the *S.marcescens* plasmid pNRT324 containing the wild type *rap* gene was also used to transform the Hor mutant ATM101. This construct too, was unable to complement the *hor*⁻ gene mutation of ATM101 (data not shown).

7.2.2 THE EFFECT OF *carR* (*in trans*) AND THE EXOGENOUS ADDITION OF OHHL ON THE HOR PHENOTYPE

Previously it had been reported that *carR* in multicopy could suppress the carbapenem negative phenotype of *Ecc* strains with a defective *carI* gene, that is, those defective in the production of the small molecule autoinducer *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL; Cox. 1995; McGowan *et al.*, 1995). To determine whether this was also true for a *hor* mutant, the multicopy plasmid pSMG4 (McGowan *et al.*, 1995) encoding the *carR* gene was used to transform ATM101. As can be seen from figure 7.5a, *carR* in multicopy suppressed the Hor phenotype, in so far as carbapenem production was restored (but the reduced exoenzyme phenotype remained unaffected, consistent with McGowan *et al.*, 1995; data not shown).

OHHL is also an integral part of the regulation of carbapenem production in *Ecc* (section 1.7.3), and therefore it was important to determine if OHHL, added exogenously, could suppress the Hor phenotype. 5µl of an overnight culture of *Ecc* strain ATM101 (*hor*) was spotted onto the *E.coli* strain ESS bioassay plate and allowed to dry in, on top of this 1µl (1mg/ml stock) of OHHL was added. Carbapenem production was indeed restored by the addition of OHHL (figure 7.5b). This result was surprising because ATM101 is not defective for the production of OHHL (as shown by crossfeeding a *Ecc carI* mutant; data not shown).

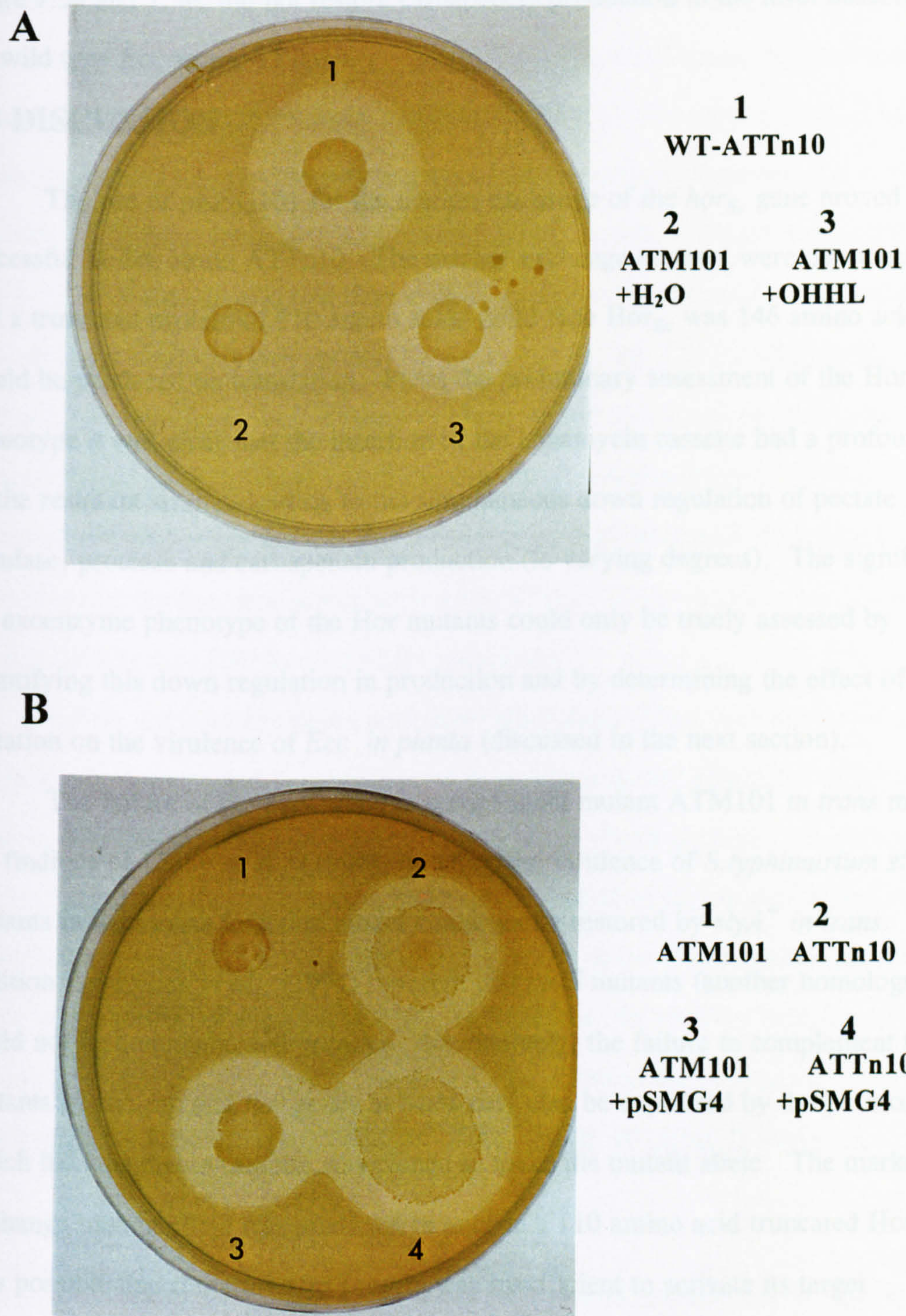
FIGURE 7.5

Figure 7.5 Analysis of the effect of exogenously added OHHL (1 μ l of 1mg/ml stock; A), and *carR*, contained on the multicopy plasmid pSMG4 (B), on the production of carbapenem by the *Ecc hor* mutant ATM101. A clear zone of antibiosis on the *E.coli* strain ESS bioassay indicates carbapenem production.

It should be noted that the apparent suppression by *carR* and OHHL, shown in figure 7.5a and 7.5b, did not restore carbapenem production to the level observed for the wild type *Ecc* strain ATTn10.

7.3 DISCUSSION

The use of pKNG101 for the marker exchange of the *hor_{Ec}* gene proved successful in *Ecc* strain ATTn10. The marker exchange mutants were constructed such that a truncated protein of 110 amino acids (wild type Hor_{Ec} was 146 amino acids) would be produced on translation. From the preliminary assessment of the Hor phenotype it was clear that the insertion of the kanamycin cassette had a profound effect on the resultant strains, leading to the simultaneous down regulation of pectate lyase, cellulase, protease and carbapenem production (to varying degrees). The significance of the exoenzyme phenotype of the Hor mutants could only be truly assessed by quantifying this down regulation in production and by determining the effect of the *hor* mutation on the virulence of *Ecc in planta* (discussed in the next section).

The failure of the *hor*⁺ gene to complement mutant ATM101 *in trans* mirrored the findings of Libby *et al.*, (1994), in which the virulence of *S.typhimurium slyA* mutants in the mouse infection model could not be restored by *slyA*⁺ *in trans*. In addition Reverchon *et al.*, (1994) reported that *pecS* mutants (another homologue of *hor*) could not be complemented *in trans*. Alternatively, the failure to complement the *hor* mutants by the *rap* and *hor* genes *in trans* may also be explained by the position into which the kanamycin cassette was cloned to make the mutant allele. The marker exchange mutant allele was predicted to encode a 110 amino acid truncated Hor protein. It is possible that this truncated protein was insufficient to activate its target genes/phenotypes but was able to interfere with the functioning of the gene products of the wild type *hor* and *rap* genes encoded *in trans*. To investigate this would require the construction of another mutant *hor* allele, for which there was insufficient time remaining for this study.

What became clear from these data was that the Hor protein was important for the production of carbapenem in *Ecc*. Carbapenem in *Ecc* is known to be regulated by a small molecule dependent regulatory system involving OHHL and the CarR protein (Bainton *et al.*, 1992a, 1992b; McGowan *et al.*, 1995). The observed effect of adding exogenous OHHL (or *carR* in multicopy) on the production of carbapenem in the *hor* mutants, when these strains already produce OHHL and have a functional *carR* gene, was difficult to explain with the data available. The suppression of a carbapenem minus phenotype by *carR* in multicopy has been reported before (Cox, 1995): CarR, when in high copy number, appears to be able to break free of its normal physiological constraints and can activate carbapenem production independently of OHHL. One of the obvious explanations for the suppression of the antibiotic null phenotype by *carR* *in trans* was that the *hor* gene affected the expression of this gene, however this would not explain the effect of OHHL on the Hor phenotype.

Before drawing any firm conclusions from these data it was important to determine if Hor affected the carbapenem biosynthetic genes directly or if it had an indirect effect on, for example, the production of the CarR protein. The following sections attempt to answer some of the questions raised so far by quantifying the observed down regulation of exoenzyme production in the Hor mutants and studying its effect on the ability of *Ecc* to cause soft rot *in planta*. In addition *lacZ* fusions were used to determine at what point the *hor* mutation affected the production of carbapenem.

7.4 DETAILED ANALYSIS OF THE HOR PHENOTYPE

7.4.1 INTRODUCTION

To make an assessment of the impact the Hor phenotype had on *Ecc*, it is first helpful to briefly review what is known about exoenzyme and antibiotic production by *Erwinia carotovora* subspecies *carotovora* (*Ecc*).

Virulence of the plant pathogenic enterobacterium *Ecc* is strongly linked to the ability to produce an array of plant tissue macerating extracellular enzymes. These

enzymes include isoforms of pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel) and protease (Prt) (Collmer and Keen, 1986; Kotoujanski, 1987).

The importance of these enzymes with respect to pathogenicity varies, with the pectate lyases being considered the most important virulence determinants (Hinton *et al.*, 1989; Collmer and Keen, 1986). This was illustrated by the fact that expression of the *Erwinia chrysanthemi* (Echr) Pel genes in *E. coli* conferred upon it a significant potato tissue macerating ability (Barras *et al.*, 1987). Cellulases are considered important in the early stages of infection (Walker, 1994a, 1994b). However the role of proteases in the pathogenicity of soft rot erwinias is equivocal (Dahler, 1990). It is clear however that mutants affected in the coordinate production of these enzymes exhibit significantly reduced virulence *in planta* (Hinton *et al.*, 1989; Murata *et al.*, 1991; Pirhonen *et al.*, 1991 and Jones *et al.*, 1993).

The regulation of exoenzyme production in these bacteria is complex, affected by both environmental and intrinsic factors (Pirhonen *et al.*, 1993; reviewed by Wharam *et al.*, 1995). Perhaps the most dramatic phenotypes with respect to the regulation of virulence are described by: Aep (activation of extracellular protein; Murata *et al.*, 1991), Exp (exoenzyme production; Pirhonen *et al.*, 1991) and the Rex mutants (regulation of exoenzymes; Jones *et al.*, 1993). All these phenotypic classes describe the global down regulation of exoenzyme production and lead to an avirulent mutant. The molecular mechanism behind these phenotypes centres on the presence of a small molecule dependent regulatory system (*N*-[3-oxohexanoyl]-L-homoserine lactone: OHHL; Bainton *et al.*, 1992a, b), analogous to that controlling bioluminescence in *Photobacterium fischeri*, as discussed in section 1.5.4 (for a review see Meighen, 1991).

Rex mutants of *Ecc* not only exhibit coordinate defects in exoenzyme production, but are also phenotypically carbapenem negative. Thus OHHL is responsible for the global regulation of both secondary metabolism and virulence determinants in *Ecc* (Jones *et al.*, 1993; McGowan *et al.*, 1995).

Ecc strains with mutations affecting secretion of these exoenzymes similarly show reduced virulence *in planta* (Chatterjee *et al.*, 1985; Murata *et al.*, 1990). Prt in *Echr* is secreted by a one step process, distinct from that of Pel and Cel, referred to as the Type I secretion pathway (Pugsley, 1993; Salmond and Reeves, 1993). Pel and Cel are secreted by the Type II secretion system, also referred to as the general secretory pathway (Salmond and Reeves, 1993). The enzymes are first exported into the periplasm by a system analogous to the *sec*-pathway of *E.coli*. Then they are secreted into the extracellular milieu by the 'Out' apparatus (Reeves *et al.*, 1993). Mutants defective in secretion accumulate Pel and Cel in the periplasmic space and are referred to as *out*⁻ mutants (Andro *et al.*, 1984).

The exoenzyme plate assays discussed in section 7.2 revealed that the *Hor* mutants were affected in both exoenzyme and antibiotic production. The aim of the following experiments was to determine whether enzyme production or secretion was affected and by how much with respect to the wild type *Ecc* strain ATTn10.

7.4.2 LIQUID ASSAYS FOR EXOENZYME PRODUCTION

To quantify the coordinate down regulation of exoenzyme production in the *hor* marker exchange mutant ATM101, assays for pectate lyases (Pel), cellulases (Cel) and proteases (Prt) were performed as described in sections 2.17.3, 2.17.2 and 2.17.1 respectively. Three replicate 50 ml side arm flasks containing 5 ml of Pel Minimal Broth (containing PGA) were set up for each strain assayed. Flasks were inoculated with 50 µl of either *Ecc* strain ATTn10 or the *Ecc hor* mutant strain ATM101, which had been standardised for optical density (OD_{600nm}) by dilution. Cultures were grown until early stationary phase (as determined from a growth curve; figure 7.6) at which point the cells were harvested and the supernatant removed and collected. The cells were sonicated (section 2.16) and the two culture fractions were assayed for enzyme activity. The difference in the enzyme activity between sonicate and supernatant

FIGURE 7.6

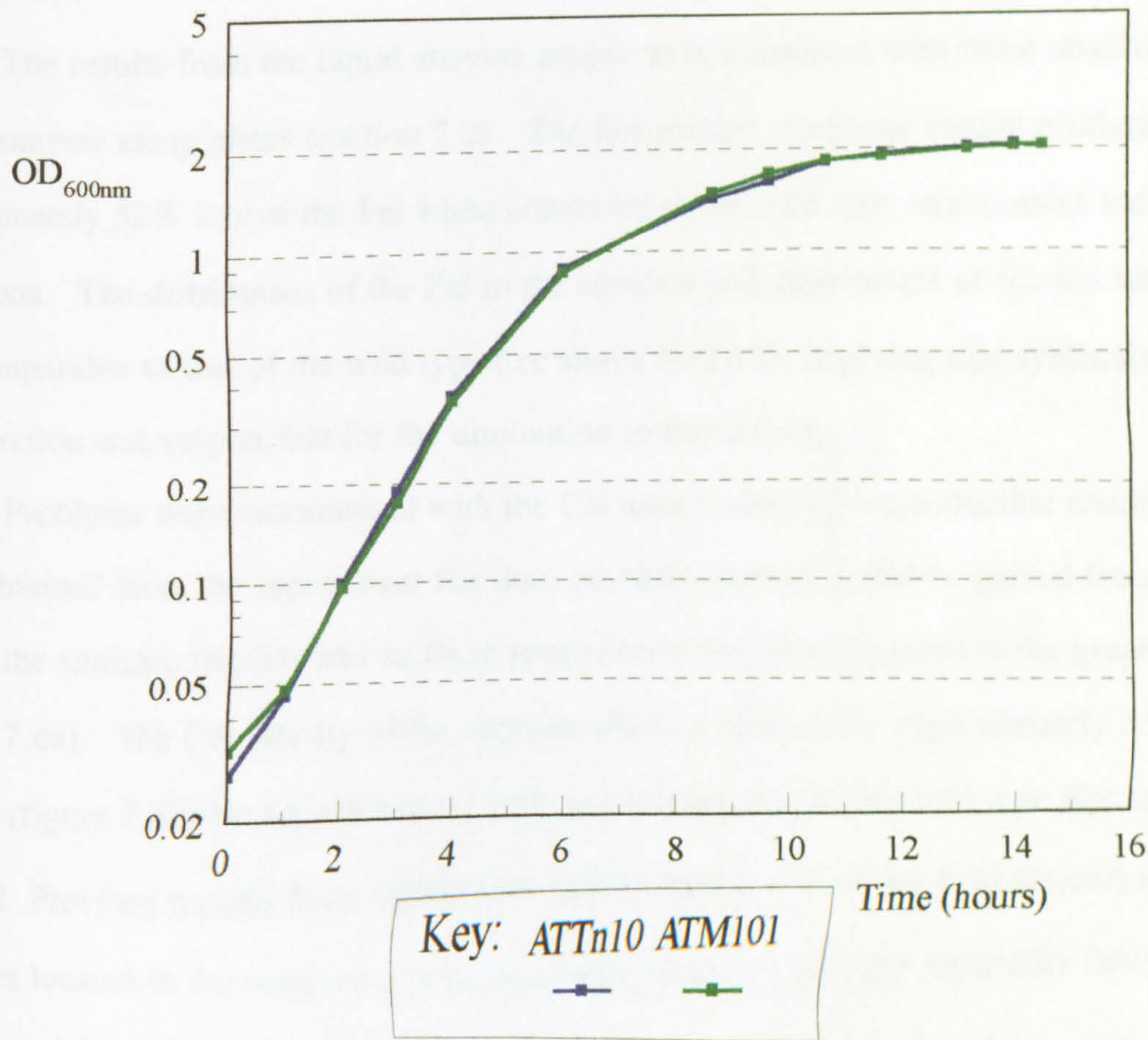


Figure 7.6 A growth curve of wild type *Ecc* strain ATTn10 and the *hor^r* marker exchange strain ATM101. Bacterial cultures were grown at 30°C in a 50 ml side arm flask and measured at time points throughout the growth curve at OD₆₀₀ on a Philips PU 8720 spectrophotometer.

fractions would indicate whether the down regulation in exoenzyme production of the *hor_{Ec}* mutant was at the level of enzyme synthesis or secretion.

Enzyme activity of Pel was measured from cells grown in Pel Minimal broth containing PGA and without PGA, ie under inducing and non inducing conditions (section 2.17.3). The results obtained for replicate cultures were pooled and are presented as graphs in figure 7.7 for Pel and figure 7.8A and B for Cel and Prt respectively.

The results from the liquid enzyme assays were consistent with those observed on the enzyme assay plates (section 7.2). The *hor* marker exchange mutant produced approximately 52% less of the Pel when compared to the wild type strain under inducing conditions. The distribution of the Pel in the sonicate and supernatant of the *hor* mutant was comparable to that of the wild type *Ecc* strain ATTn10, implying that synthesis and not secretion was responsible for the diminution in Pel activity.

Problems were encountered with the Cel assay: although reproducible results were obtained from the supernatant fraction, no such readings could be gained from testing the sonicate fraction and so these results have not been included in the graph (figure 7.8a). The Cel activity of the supernatant was reduced by approximately 33% and Prt (figure 7.8b) by approximately 20% when compared to the wild type *Ecc* strain ATTn10. Previous reports have shown that approximately 1% of the total exoenzyme activity is located in the sonicate of wild type cells (Jones, 1994) the remainder being secreted and found in the supernatant. This agrees well with the results of this study where protease activity detected in the sonicate for ATM101 was approximately 1.3 % of the total activity, suggesting secretion of Prt was also not affected by the *hor* gene mutation.

FIGURE 7.7

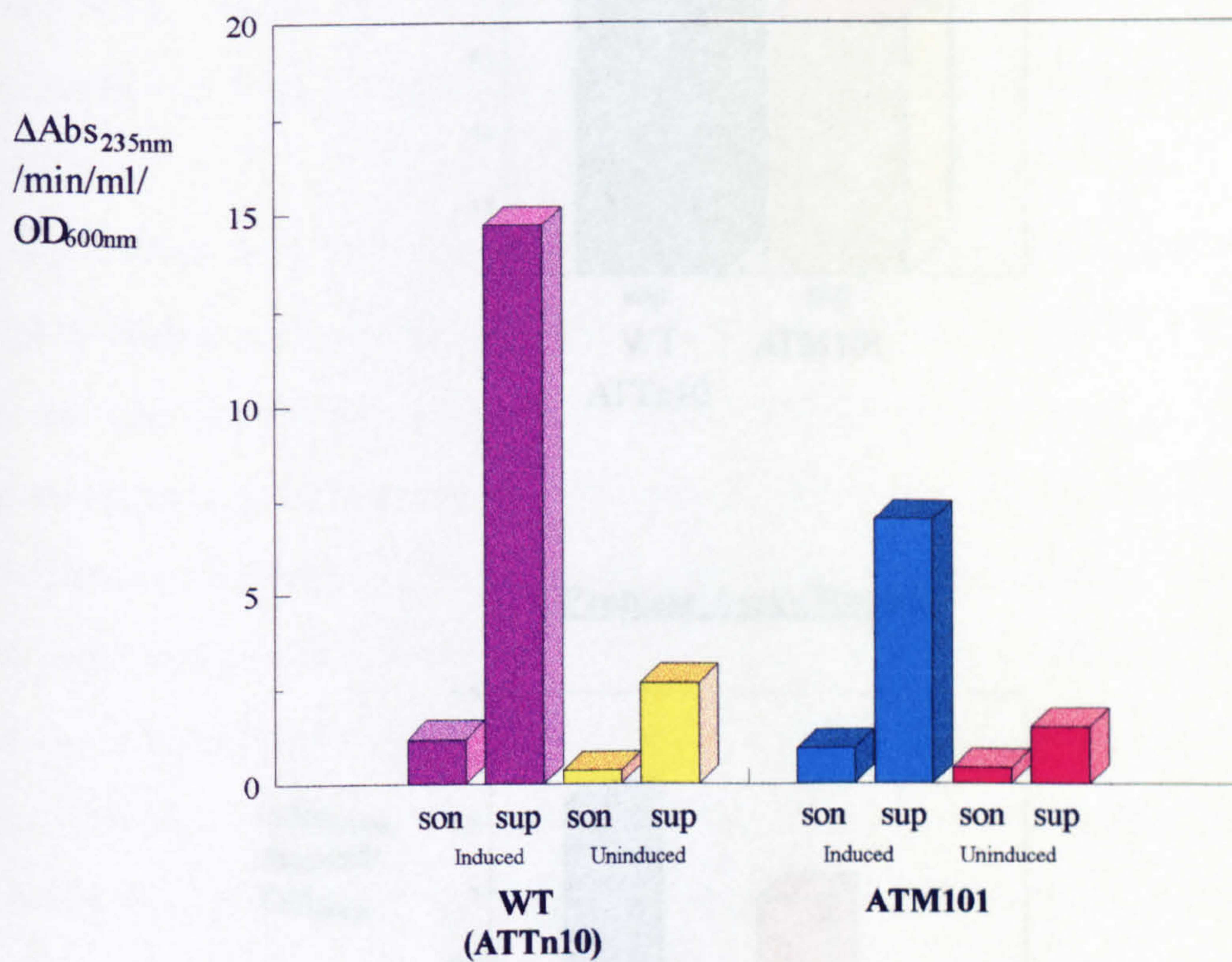


Figure 7.7 The results of liquid enzyme assays for pectate lyase production by the *Ecc hor* mutant **ATM101**. The bar graph shows the total production of pectate lyases as the cells enter early stationary phase (14 hours into the growth curve), as defined by liquid enzyme assays based on the method of Starr *et al.*, (1977). Results are expressed as $\Delta\text{abs}_{235} / \text{min/ml} / \text{OD}_{600}$ unit. The fractionation control was that at least 89% of β -galactosidase activity was located in the sonicate. (Key: Sup- supernatant; Son- sonicate; Induced - cells grown in inducing medium [Pel minimal plus PGA]; Uninduced -cells grown in pel minimal).

FIGURE 7.8

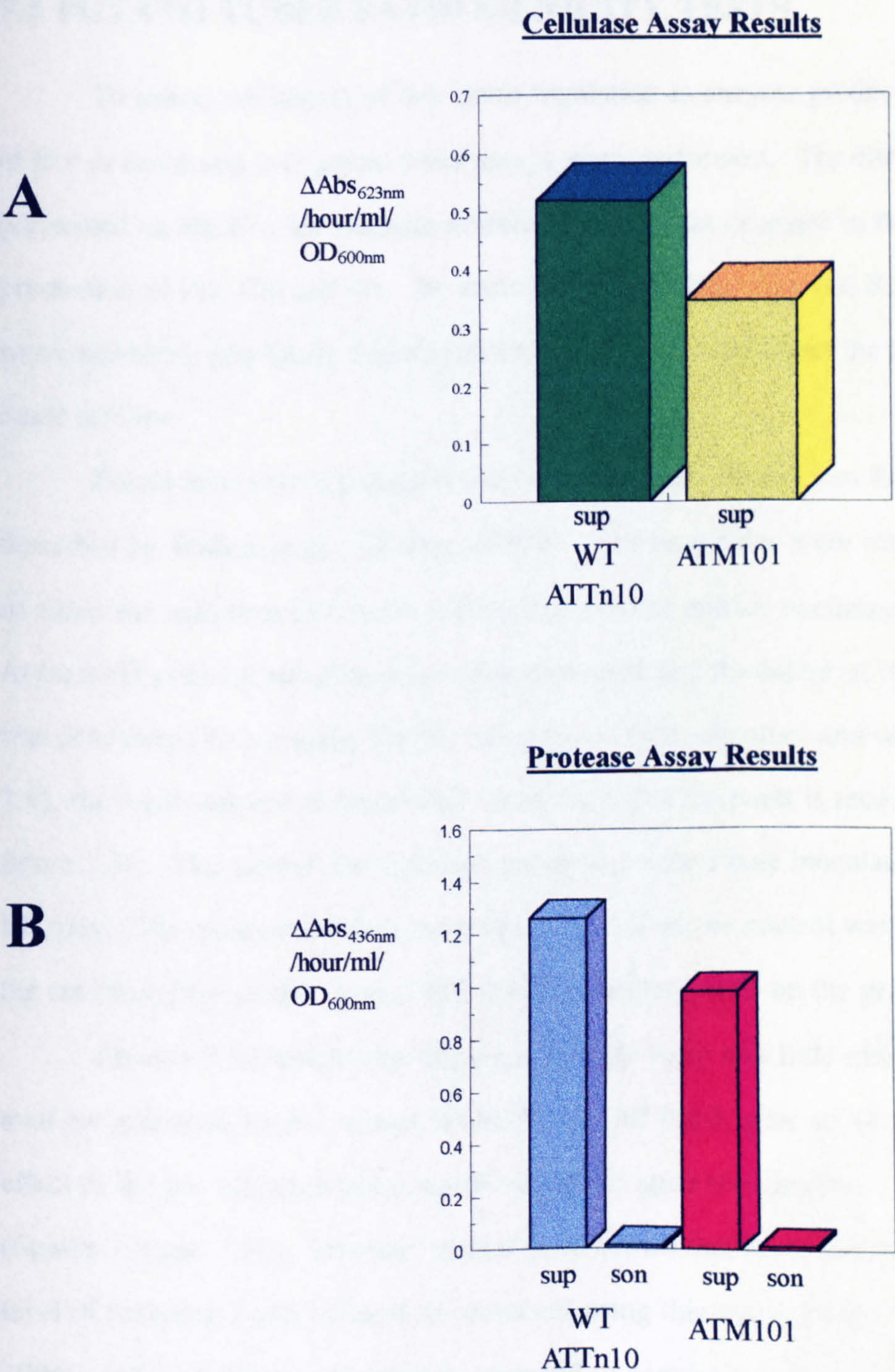


Figure 7.8 The results of liquid enzyme assay for cellulase and protease production by the *Ecc hor* mutant ATM101. The bar graphs show the total production of cellulase (Cel; **A**) and protease (Prt; **B**) as the cells enter early stationary phase (14 hours into the growth curve), defined by liquid enzyme assays based on the method of Nelson, (1944) and Samogyi, (1952) for Cel and Braun and Schmitz, (1980).for Prt. The fractionation control was that at least 89% of β -galactosidase activity was located in the sonicate. (Key: sup- supernatant; son- sonicate).

7.5 POTATO TUBER PATHOGENICITY TESTS

To assess the impact of this down regulation in enzyme production on the ability of *Ecc* to cause soft rot, potato tuber assays were performed. The enzyme assays performed on the *Ecc hor* mutants revealed a significant decrease in the coordinate production of Pel, Cel and Prt. By analogy with the Aep, Exp and Rex mutations, it was considered very likely that this down regulation would affect the ability of *Ecc* to cause soft rot.

Potato tubers were prepared and two holes were drilled into them as previously described by Walker *et al.*, (1994 a, 1994b). The bore holes were inoculated with 10 µl of either the wild type *Ecc* strain ATTn10 or the *hor* marker exchange mutant ATM101. At least six potatoes per time point were harvested and the extent of tissue maceration was determined by scraping out the rotten tissue from the tuber and weighing it (figure 7.9), the mean amount of macerated tissue for each time point is recorded as a graph in figure 7.10. The control for this experiment was a bore hole inoculated with 10 µl of LB only. The mean amount of macerated tissue from the control was subtracted from the rot caused by the *Ecc* strains tested before being plotted on the graph.

Figures 7.10 reveals that although initially there was little difference between the total rot generated by *Ecc* strains ATM101 and ATTn10, after an extended period the effect of the *hor* mutation had a significant effect upon the virulence of this strain (figures 7.9 and 7.10). Previous studies have shown that there can be a pronounced level of variation in the level of rot observed using this assay (Jones, 1994: Walker, 1994a) and so further tests would be required to conclusively assess the reduced virulence phenotype of ATM101.

FIGURE 7.9

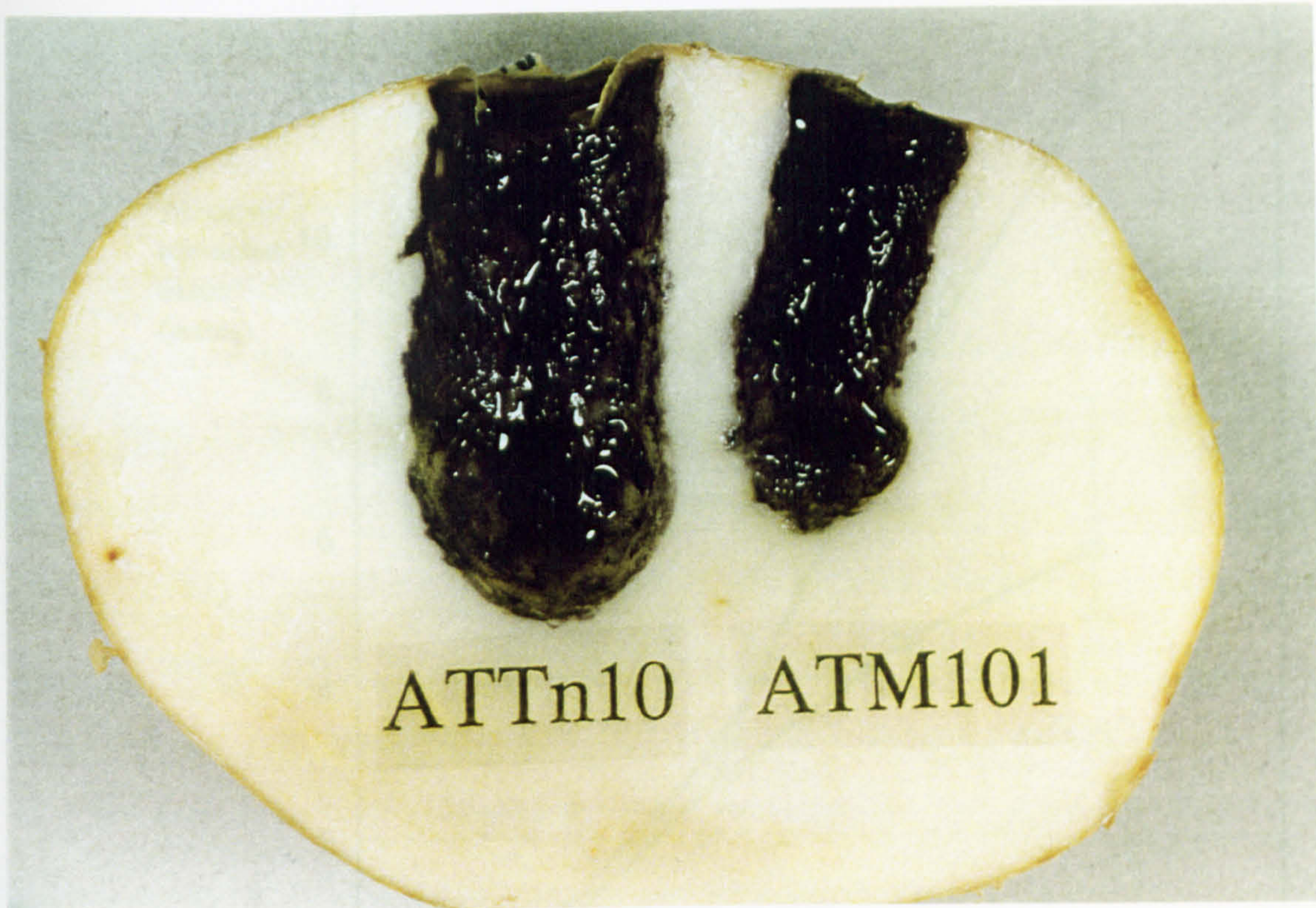
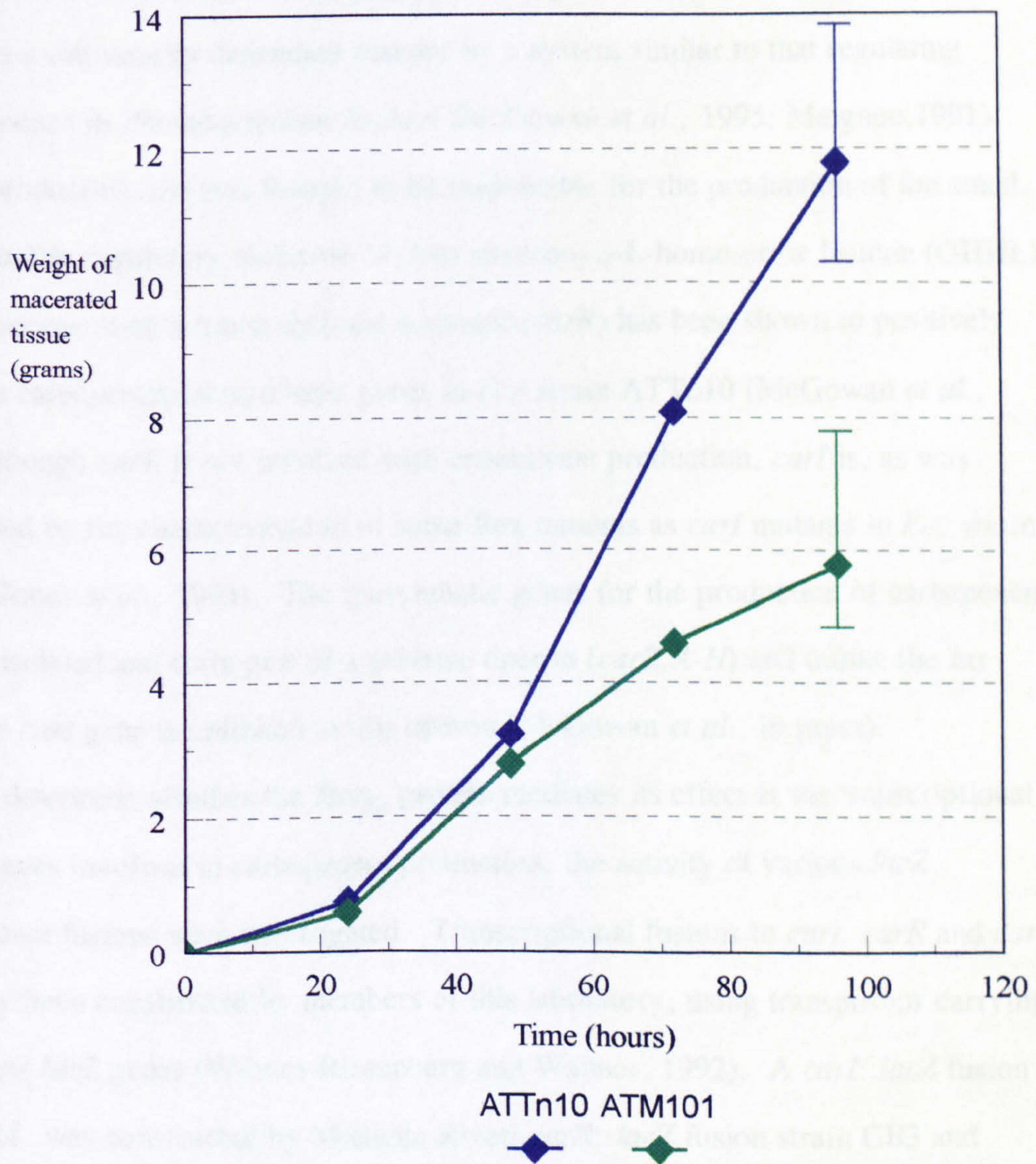


Figure 7.9 A potato tuber used to assay virulence of the *hor* marker exchange mutant ATM101

The potato tuber (cultivar: Pentland Javelin) has been cut through the centre of the inoculation sites and the macerated tissue has been removed. The tuber was stained with iodine for this photograph to aid visualisation. The wild type *Ecc* strain was ATTn10 (**left**) and the *hor* marker exchange mutant used was ATM101 (**right**).

FIGURE 7.10**Figure 7.10 Potato tuber virulence assays.**

Potatoes were inoculated with 10 μ l of a liquid culture of either the wild type *Ecc* strain ATTN10 or the *hor* marker exchange mutant ATM101. Potato tubers were harvested at intervals over a 96 hour period and the extent of the rot determined for each time point. The plotted points represent the mean of the total rot for each strain from six replicate potato tubers. The variation in rotting between tubers is represented by error bars (for the last time point only).

7.6 INVESTIGATION OF CARBAPENEM PRODUCTION BY THE *Ecc hor* MUTANTS

7.6.1 INTRODUCTION

The plate assays and HPLC analysis (section 7.2) revealed that the *hor* mutants produced no detectable level of carbapenem. Carbapenem in *Ecc* is known to be regulated in a cell density dependent manner by a system similar to that regulating bioluminescence in *Photobacterium fischeri* (McGowan *et al.*, 1995; Meighen, 1991). The gene product of *carI* it is thought to be responsible for the production of the small, highly diffusible regulatory molecule *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL). OHHL in concert with a transcriptional activator (*carR*) has been shown to positively activate the carbapenem biosynthetic genes in *Ecc* strain ATTn10 (McGowan *et al.*, 1995). Although *carR* is not involved with exoenzyme production, *carI* is, as was demonstrated by the characterisation of some Rex mutants as *carI* mutants in *Ecc* strain SCRI193 (Jones *et al.*, 1993). The biosynthetic genes for the production of carbapenem have been isolated and form part of a putative operon (*carR, A-H*) and unlike the *lux* system, the *carI* gene is unlinked to this operon (McGowan *et al.*, in press).

To determine whether the Hor_{Ec} protein mediates its effect at the transcriptional level, on genes involved in carbapenem production, the activity of various *lacZ* transcriptional fusions were investigated. Transcriptional fusions to *carI*, *carR* and *carA* had already been constructed by members of this laboratory, using transposons carrying promoterless *lacZ* genes (Wilmes-Riesenberg and Wanner, 1992). A *carI::lacZ* fusion strain, MR1, was constructed by Michelle Rivet; *carR::lacZ* fusion strain GB3 and *carA::lacZ* fusion strain GB7 were constructed by Gulgan Bozgelmez. To study the effect of the *hor* gene mutation on the expression of these genes, phage ϕ KP (Toth, 1991; Toth *et al.*, 1993) was used for strain construction, as discussed in the next section.

7.6.2 CONSTRUCTION OF *hor* MUTANT STRAINS WITH *LacZ* TRANSCRIPTIONAL FUSIONS TO *carI*, *carR* AND *carA* GENES

Construction of the dual *hor*⁻ / *lacZ* fusion strains was achieved by producing a ϕ KP lysate on the marker exchange *Ecc* strain ATM101. The resultant lysate was called ϕ KP-ATM101 and was used to transduce three *Ecc* strains (*Lac*⁻ strain but with *Lac*⁺ gene fusion), namely MR1, GB3 and GB7 as previously described (section 2.11). Transductants were selected on NBA supplemented with kanamycin, to select for the *hor::kan*^R allele.

Several kanamycin resistant transductants from each of these experiments were grown up overnight in NB and the chromosomal DNA was extracted and cut with restriction enzymes *MluI* and *SalI* in preparation for a Southern blot (figure 7.11a). As discussed in section 7.1.2 these enzymes do not cut in the kanamycin cassette and so if the transduction was successful, probing with a *hor* gene probe would result in a band shift. The probe used in the Southern blots was a 597 bp DNA fragment, amplified by PCR and DIG labelled, which encoded the putative *hor* gene. The primers used to make this probe were Cyclcyto1 and Cyclcyto2 (appendix) with an annealing temperature of 44°C. All other parameters have been described previously (section 2.6).

The results for the Southern blot of the *hor*⁻ / *carI::lacZ* fusion strains, are presented in figure 7.11b. A band shift of approximately 1266 bp (equal to the size of the kanamycin cassette) was observed, indicating that the strain construction had been successful. One of these transductants was selected for all further experiments and was designated MRH1. The other two strains were also successfully transduced with ϕ KP-ATM101, as determined by Southern blot (data not shown) and were subsequently designated GBH3 and GBH7, derivatives of *Ecc* strains GB3 (*carR::lacZ*) and GB7 (*carA::lacZ*) respectively.

To determine the effect of the *hor* gene mutation on the transcription of these genes, β -galactosidase activity was monitored throughout the growth cycle and compared to the wild type *Ecc* strain as discussed below.

FIGURE 7.11a.

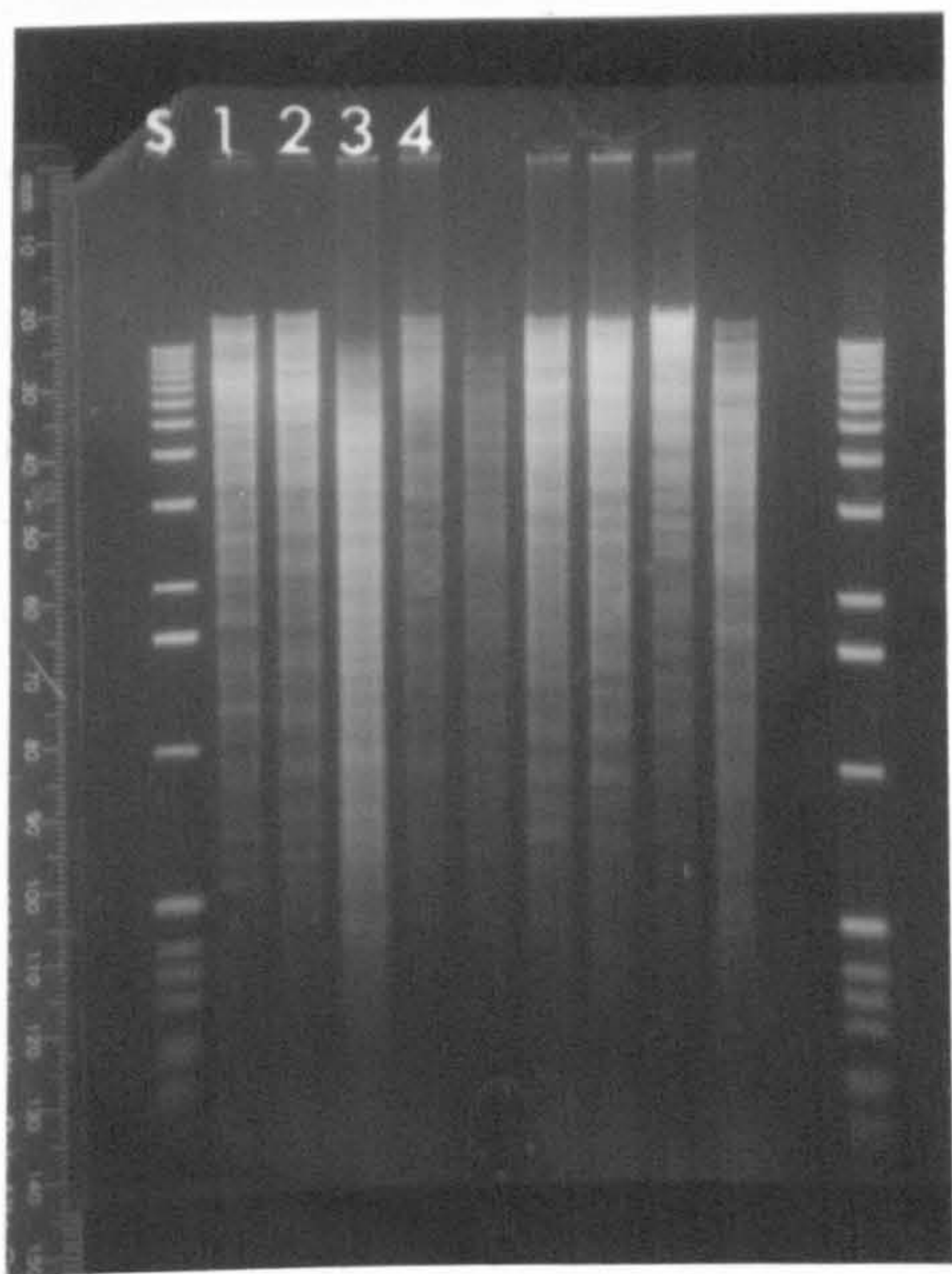


Figure 7.11a Restriction enzyme digests of *carI::lacZ* transcriptional fusion *Ecc* strain MR1 transduced with lysate Φ KP-ATM101. Chromosomal DNA from the MR1 transductants (transduced with ϕ KP-ATM101) cut with restriction enzymes *Mlu*I - *Sal*I

Key to figures 7.11a and 7.11b		
Chromosomal DNA from:-		
lane		
S		1kb ladder
1		Wild type <i>Ecc</i> strain ATTN10
2	Mutant	MRH1-1
3	"	MRH1-2
4	"	MRH1-3

7.6.3 β -GALACTOSIDASE ASSAYS FOR THE *Ecc hor* / *lacZ* FUSION STRAINS

The level of β -galactosidase activities of the *hor*⁻ fusions strains MRH1, GBH3 and GBH7 (section 7.6.2) were determined by the method of Miller *et al.*, (1972). Supernatant and cellular culture fractions were pooled before fractionation to determine the total β -galactosidase activity throughout the growth curve (section 2.16 and 2.17.4).

The results obtained from section 7.2.2 indicated that addition of exogenous OHHL could to some extent suppress the carbapenem null phenotype of a *hor* mutant. To test this more accurately, four cultures of GBH7 were set up. OHHL was added (final concentration of 1 μ g/ml) to two of the GBH7 flasks, one at the beginning (T=0) of the experiment and another after 4 hours of incubation (T=4). The β -galactosidase activities for the GB7 and GBH7 cultures are plotted in figure 7.12 and those for MRH1 and GBH3 are detailed in figures 7.13 and 7.14 respectively. *Ecc* fusion strains MR1, GB3 and GB7 were used as references to compare the effect of the *hor* gene mutation on the expression of the relevant gene.

7.6.4 ANALYSIS OF THE RESULTS FOR THE β -GALACTOSIDASE ASSAYS

The results for the *lacZ* fusions to *carR* and *carI* (figures 7.13 and 7.14) can be summarised very easily: the β -galactosidase activity of these *hor*⁻ strains (GBH3 and MRH1) was almost identical to the profiles of the parental *hor*⁺ *lacZ* fusion strains, GB3 and MR1 respectively. However the β -galactosidase profile for *Ecc* strain GBH7 was very different from its *hor*⁺ progenitor -*Ecc* strain GB7 (*carA::lacZ*). The highest β -galactosidase activity of GBH7 was less than 1% of the equivalent recorded for GB7. The high level of β -galactosidase activity generated by GB7 at the start of the growth curve (figure 7.12a) probably resulted from the residual activity derived from the inoculum itself.

The addition of exogenous OHHL could, to a limited degree, suppress the Hor phenotype (with regard to carbapenem production). The maximum activity reached for

FIGURE 7.11b

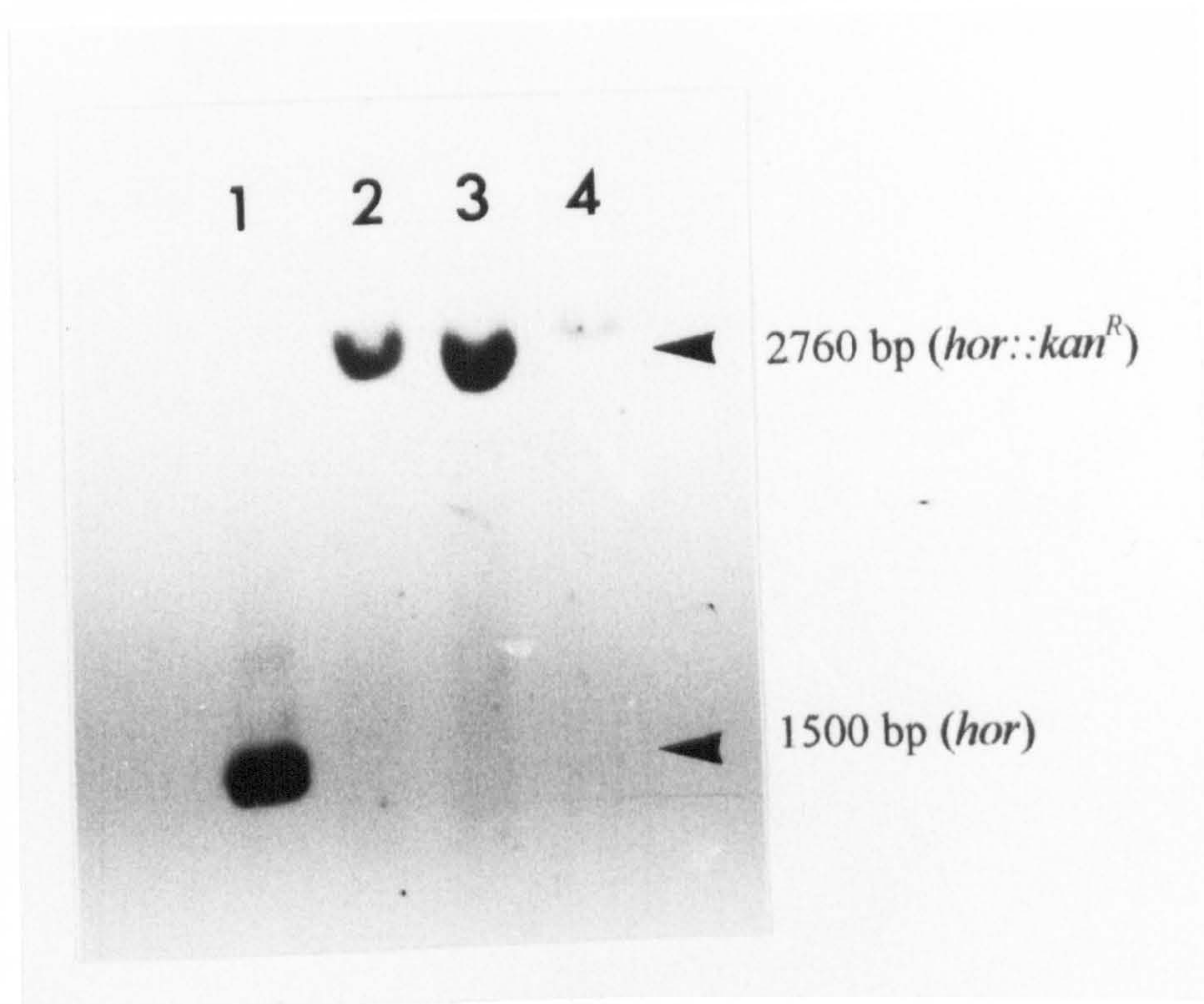
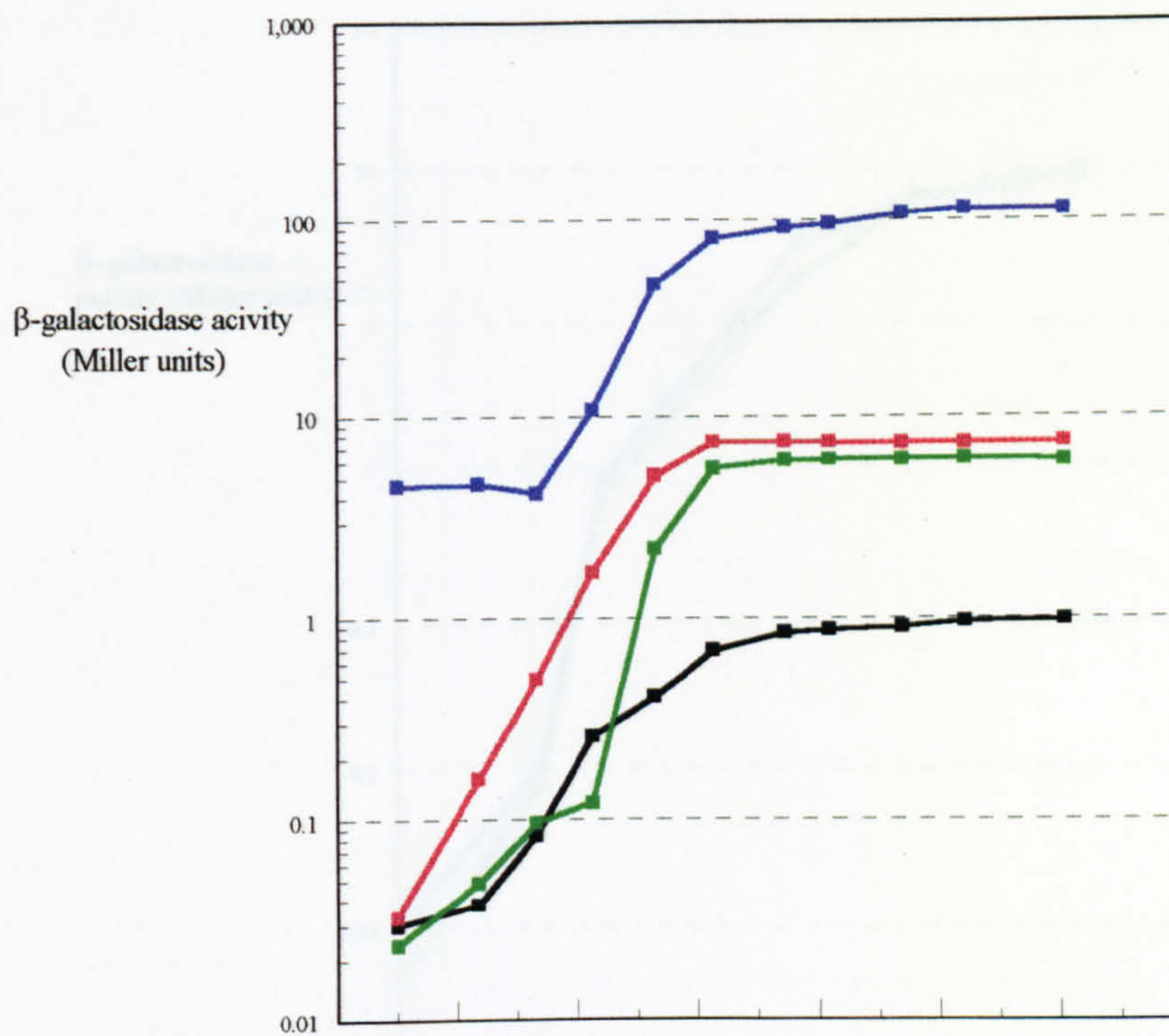


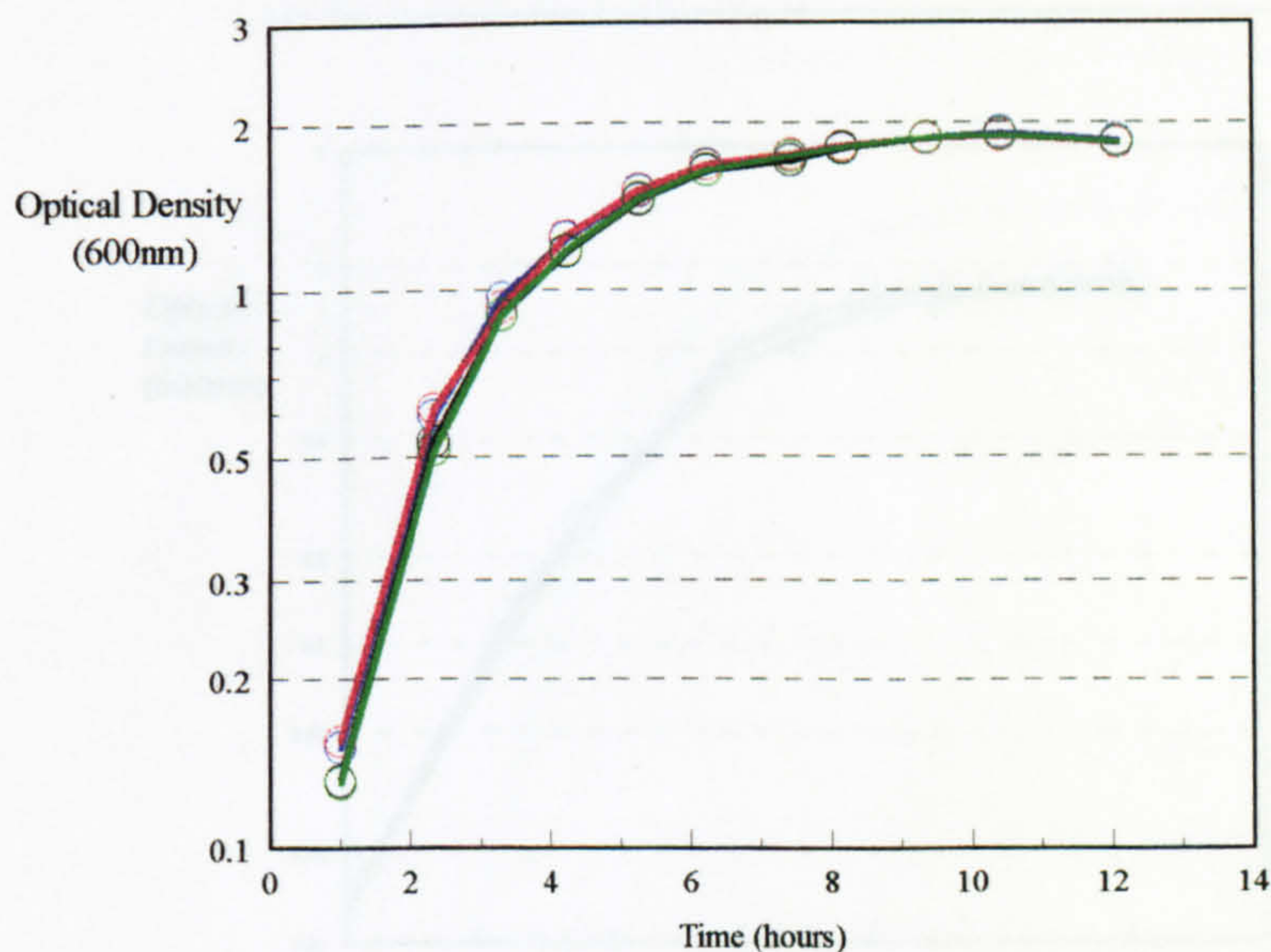
Figure 7.11b Southern blot to confirm the construction of the *hor*⁻ / *carI::lacZ* transcriptional fusion strain MRH1. The MR1 *carI::lacZ* *Ecc* strains which were transduced with lysate ϕ KP-ATM101 have been designated MRH1 numbers 1-3. The Southern blot was probed with a 597 bp *hor* DIG probe made by PCR. The hybridisation temperature for this blot was 55°C. The ATTh10 wild type exhibited a hybridising band of ~1500 bp. All the 'marker exchanged' strains exhibited hybridising bands of ~2760 bp, equal to the *MluI*-*SalI* band containing the *hor::kan^R* cassette.

FIGURE 7.12

A



B

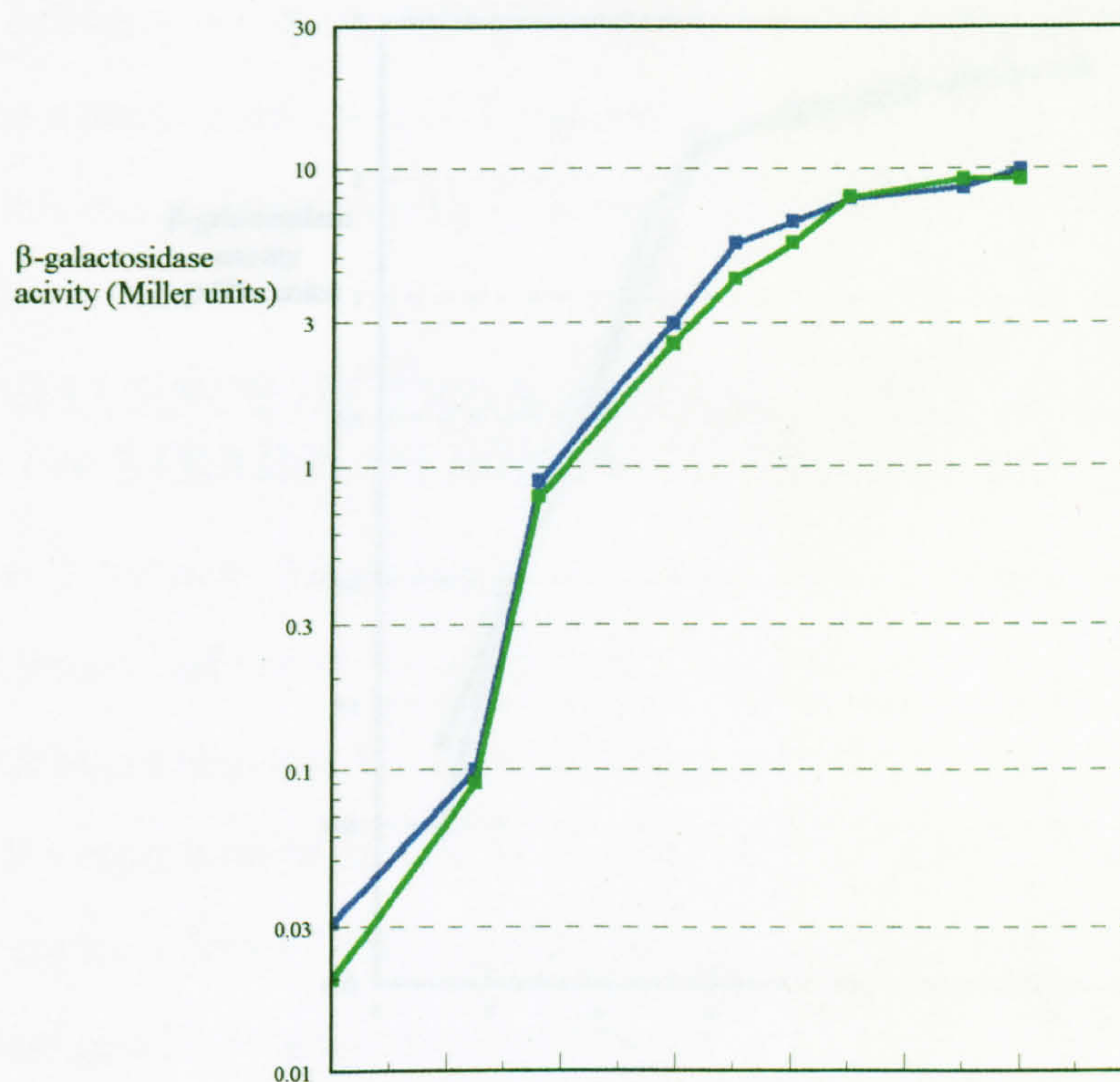


Key: GB7 GBH7 GBH7 GBH7
(T=0) (T=4)

Figure 7.12 β-Galactosidase activity of a *carA::lacZ* fusion measured in both *hor*⁺ and *hor*⁻ background. β-galactosidase activity was measured throughout the growth curve, by the method of Miller *et al.*, (1972) and are shown in Miller units (A). Strain GB7 is *hor*⁺ and GBH7 *hor*⁻. To look at the effect of OHHL on the transcription of *carA* in a *hor* mutant, OHHL was added at the start (T=0) and after four hours (T=4) of starting the experiment (see text). The growth curve (B) was drawn measuring the OD₆₀₀ on a Philips PU 8720 spectrophotometer.

FIGURE 7.13

A



B

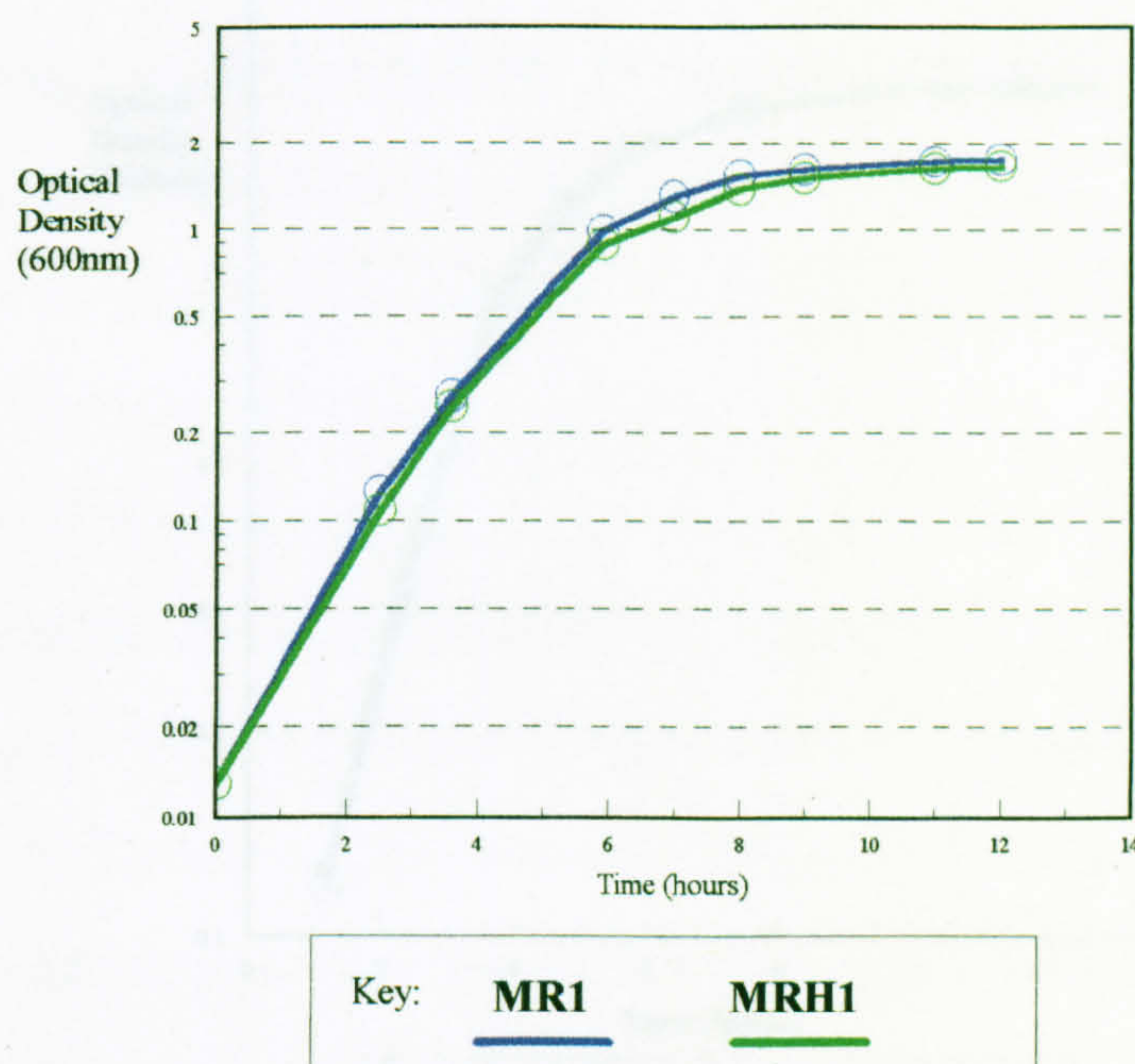
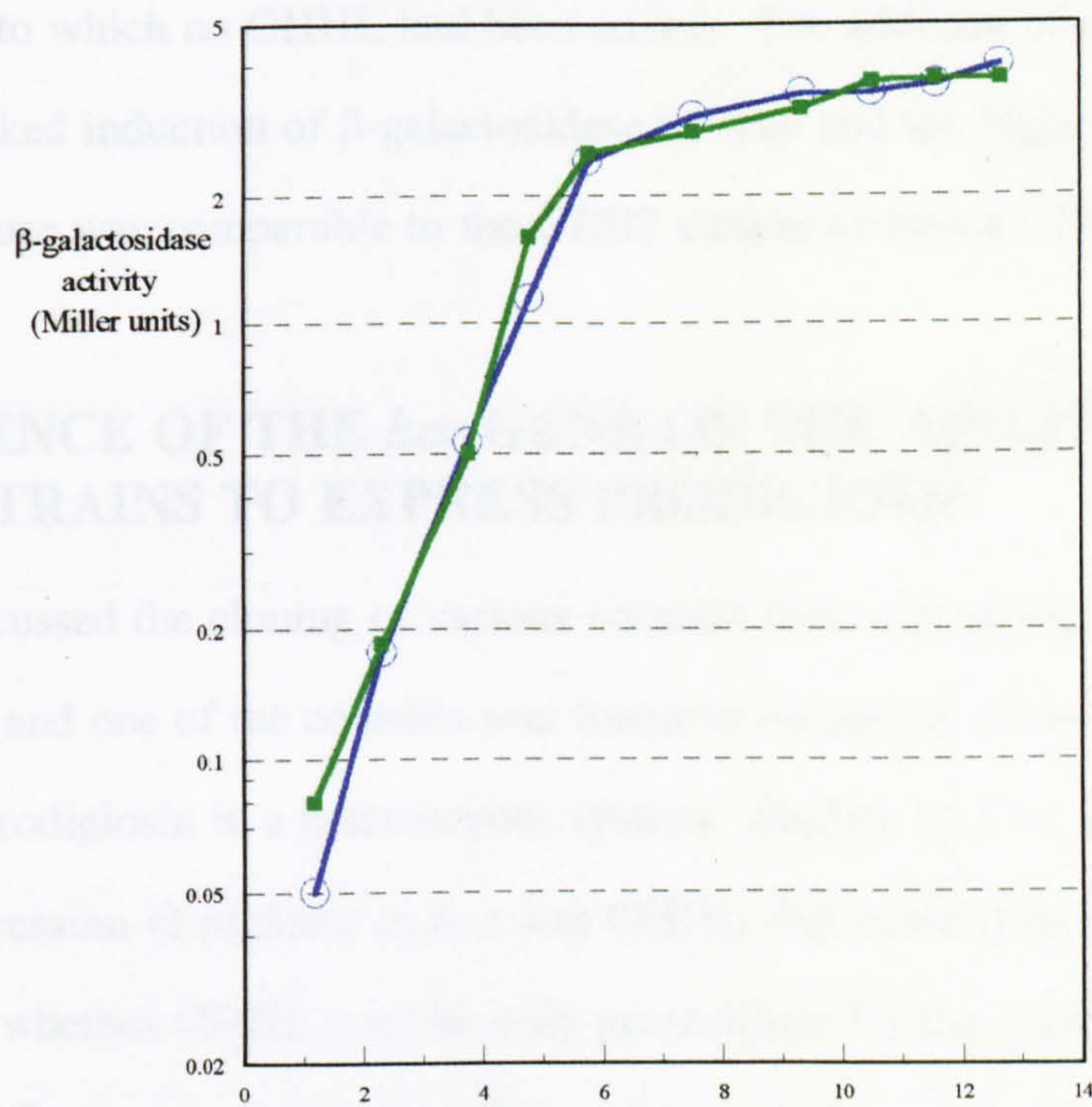


Figure 7.13 β-Galactosidase activity of a *carI::lacZ* fusion measured in both *hor*⁺ and *hor*⁻ background. β-galactosidase activity was measured throughout the growth curve by the method of Miller *et al.*, (1972) and are shown in Miller units (A). Strain MR1 is *hor*⁺ and MRH1 *hor*⁻. The growth curve (B) was drawn measuring the OD₆₀₀ on a Philips PU 8720 spectrophotometer.

FIGURE 7.14

A



B

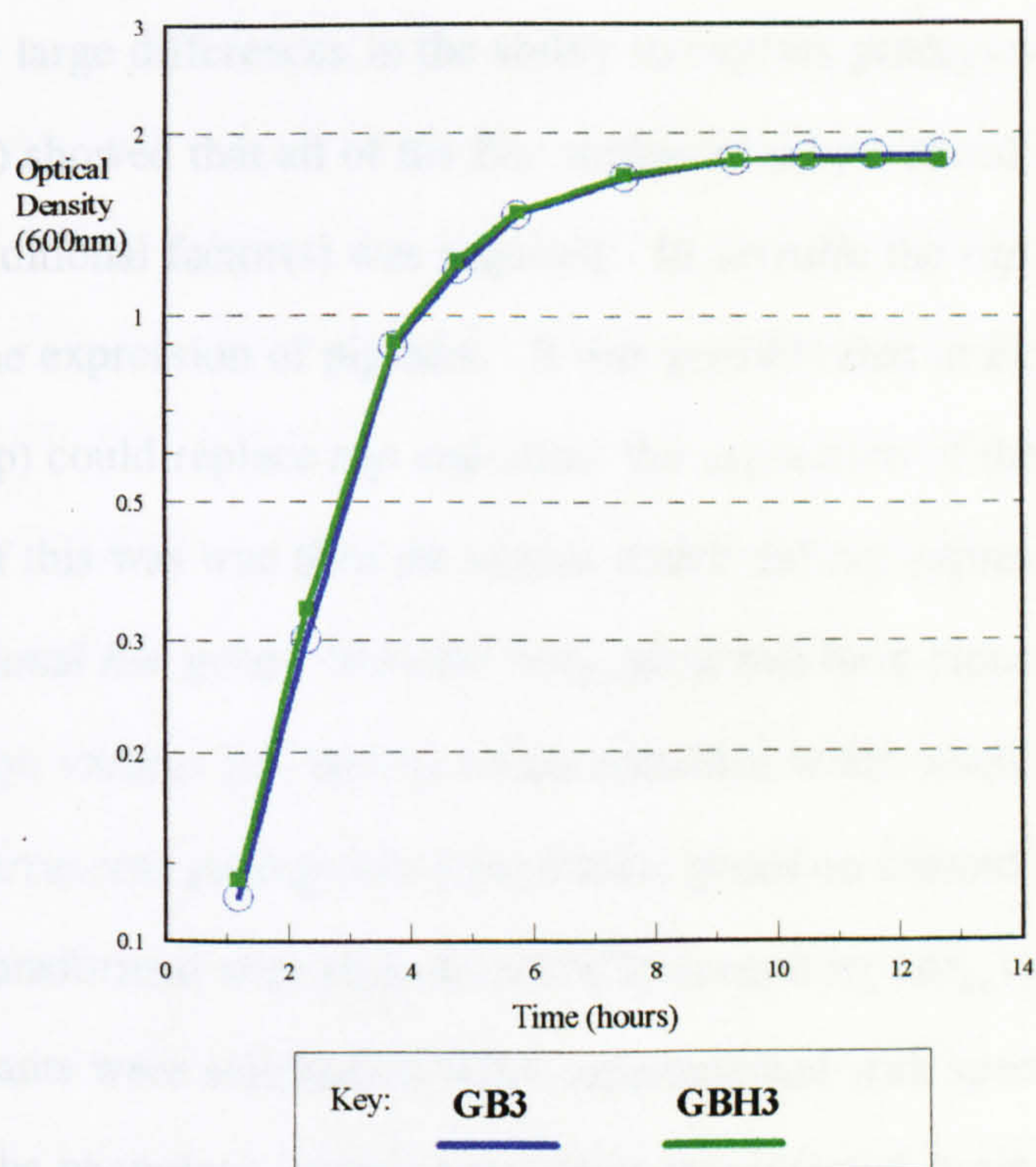


Figure 7.14 β -Galactosidase activity of a *carR::lacZ* fusion measured in both *hor*⁺ and *hor*⁻ background. β -galactosidase activity was measured throughout the growth curve by the method of Miller *et al.*, (1972) are shown in Miller units (A). Strain GB3 is *hor*⁺ and GBH3 is *hor*⁻. The growth curve (B) was drawn measuring the OD₆₀₀ on a Philips PU 8720 spectrophotometer.

the GBH7 culture to which OHHL was added at time=0 was approximately 6% of the equivalent activity detected for GB7. This represented an increase of >5% above that of the GBH7 culture to which no OHHL had been added. The addition of OHHL at T=4 produced a marked induction of β -galactosidase activity and the highest activity recorded for this culture was comparable to the GBH7 culture to which OHHL had been added at T=0.

7.7 THE INFLUENCE OF THE *hor* GENE ON THE ABILITY OF VARIOUS *Ecc* STRAINS TO EXPRESS PRODIGIOSIN

Chapter 3 discussed the cloning of various cosmids from a *S.marcescens* chromosomal library and one of the cosmids was found to encode all of the information required to express prodigiosin in a heterologous system. Studies by Cox, (1995) revealed that the expression of pigment in *Ecc* was OHHL dependent (figure 3.2). In an attempt to determine whether OHHL was the only prerequisite for the expression of this pigment, cosmid pNRT104 was used to transform a large number of *Ecc* strains revealing that there were large differences in the ability to express prodigiosin. The results of Holden, (1996) showed that all of the *Ecc* strains tested produced OHHL and so it was clear that an additional factor(s) was required. In *Serratia* the *rap* gene had been found to regulate the expression of pigment. It was possible that in *Ecc* the *hor_{Ec}* gene (a homologue of *rap*) could replace *rap* and allow the expression of the *Serratia* pigment genes in *Ecc*. If this was true then the strains which did not express prodigiosin may have lacked a functional *hor* gene. Now the *hor_{Ec}* gene had been cloned it was possible to test this theory: various *Ecc* strains which remained white when transformed previously with the *S.marcescens* prodigiosin biosynthetic genes on cosmid pNRT104 (section 3.1), were co-transformed with plasmid pNTC25 (encoding *hor_{Ec}*) and cosmid pNRT104 and transformants were selected on NBA supplemented with spectinomycin and chloramphenicol. The phenotype of the successfully transformed strains is recorded in table 7.1.

FIGURE 7.15

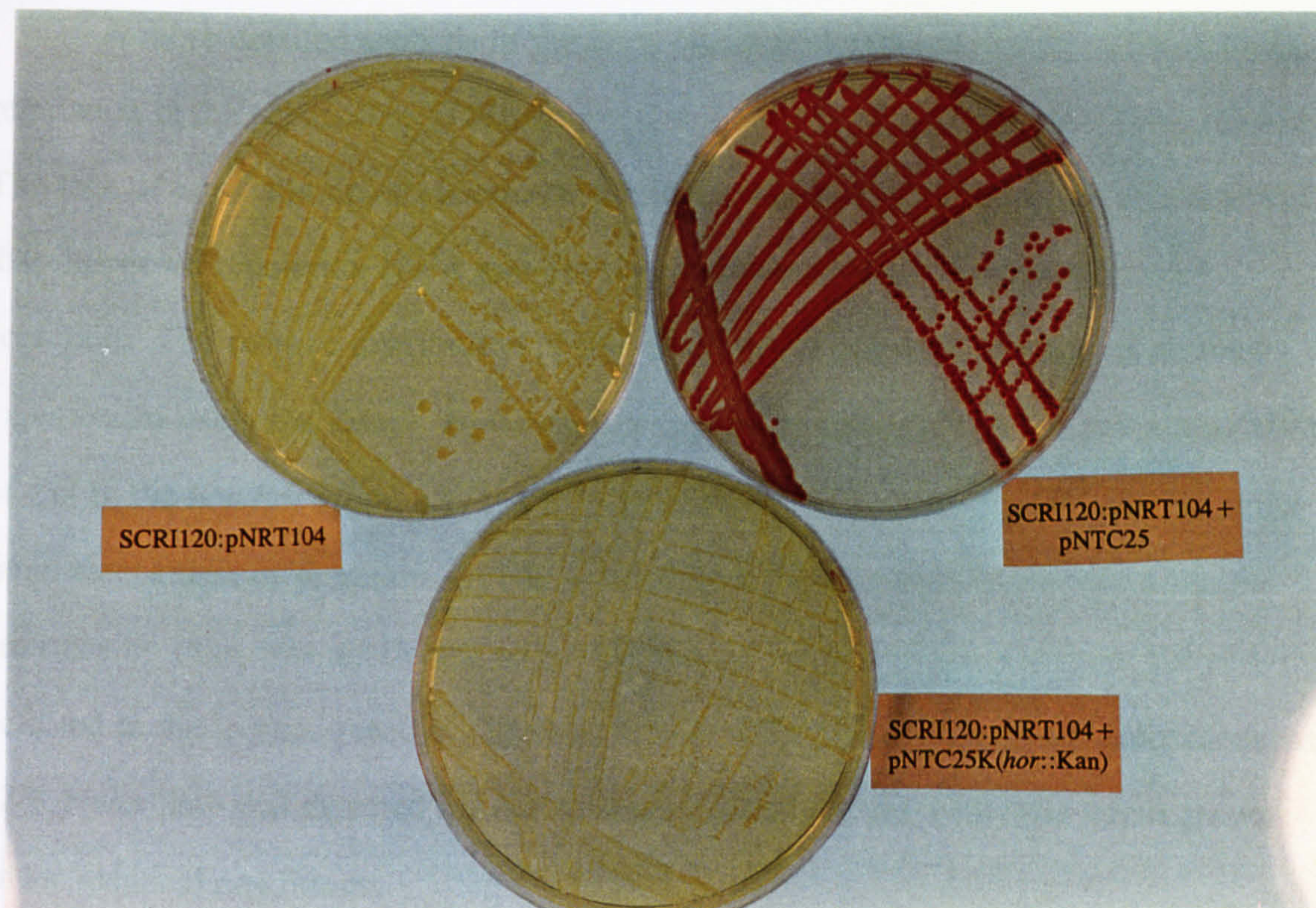


Figure 7.15 Expression of prodigiosin by SCRI120 transformed with pNRT104 and pNTC25
Ecc strain SCRI120 transformed with the prodigiosin biosynthetic cosmid pNRT104 (**top left**), pNRT104 (prodigiosin biosynthetic genes) and pNTC25 (wild type *hor* gene; **top right**) or pNRT104 and pNTC25K (*hor::kan^R*; **bottom**).

FIGURE 7.16

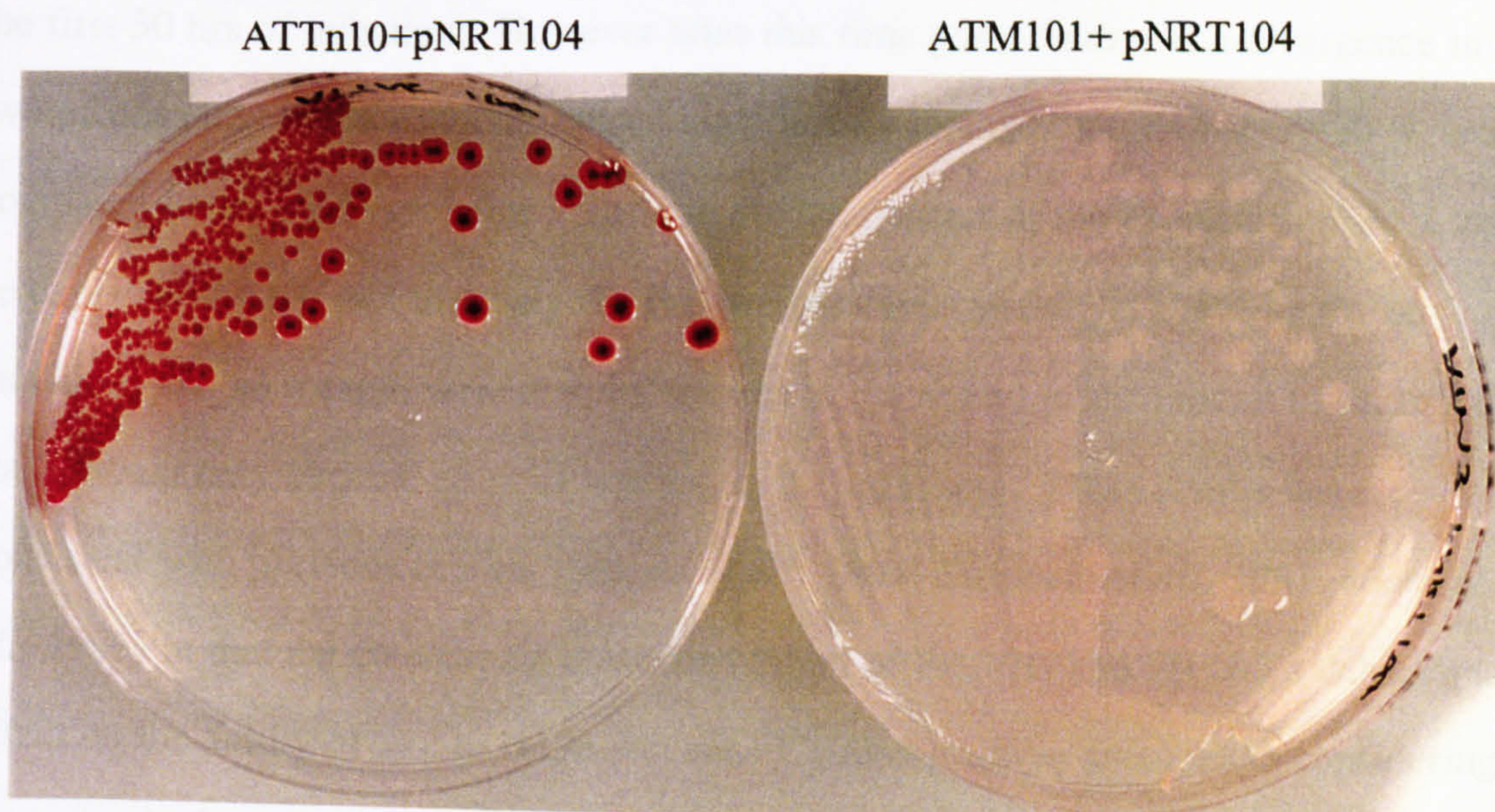


Figure 7.16. The wild type (*hor⁺*) *Ecc* strain ATTn10 (left) and the (*hor⁻*) marker exchange *Ecc* strain ATM101 (right) both transformed with pNRT104; the prodigiosin biosynthetic cosmid.

TABLE 7.1

<i>Ecc</i> strain	+ pNRT104	+ pNRT104 & pNTC25
SCRI102	White	White
SCRI106	White	White
SCRI115	White	Red
SCRI120	White	Red
SCRI121	White	a
SCRI149	White	White
SCRI198	White	White

Table 7.1 Analysis of the expression of prodigiosin in *Ecc* strains with *hor* in trans
The table shows the phenotype of various *Ecc* strains when transformed with the cosmids listed across the top; plasmid pNRT104 encodes the putative prodigiosin biosynthetic genes; pNTC25 encodes the *Ecc hor* gene. Key: Red- the expression of prodigiosin pigment; a- transformant with this set of plasmid/cosmids was not isolated. The strains used in this experiment were previously characterised as being white when transformed with the cosmid pNRT104 in section 3.1.

As can be seen from table 7.1, *hor_{Ec}* in trans facilitated the expression of pigment in *Ecc* strains SCRI120 (figure 7.15) and SCRI115. To confirm that the *hor_{Ec}* gene was responsible for this new found ability to express pigment, SCRI120 was co-transformed with pNRT104 and pNTC25K. Plasmid pNTC25K contained an inactivated *hor_{Ec}* gene constructed for marker exchange (section 7.1.1). The resulting transformants were no longer able to express prodigiosin as shown in figure 7.15.

Following on from these experiments, if *hor_{Ec}* was important for the expression of prodigiosin by *Ecc* then it is apparent that the marker exchange mutants should not be able to express this pigment. To investigate this the marker exchange mutant ATM101 was transformed with pNRT104 and transformants were selected on NBA supplemented with spectinomycin. The resulting transformant failed to express pigment to the level of the wild type control ATTn10 (figure 7.16).

7.8 SUMMARY AND DISCUSSION

A more detailed analysis of the *hor_{Ec}* mutants confirmed that there was a down regulation in the production of exoenzymes, with Pel being affected most dramatically. The lack of any build up of exoenzymes within the cells (sonicated fractions) indicated that this down regulation of Prt and Pel was at the level of enzyme synthesis not secretion. The relative distribution of Cel could not be assessed because of problems experienced using the assay. However it is extremely unlikely that the down regulation of Cel in the *hor Ecc* strain was at the level of secretion because Pel and Cel share the same mechanism of secretion. It was interesting to note that the induction of the Pel enzymes by PGA was unaffected in the *hor* mutant, with significantly more Pel activity detected in the inducing media. The maximal level of Pel activity in the supernatant was 52% lower than that detected for the analogous sample of the wild type strain grown under identical conditions.

Pel isozymes are considered to be the most important virulence determinants in the *Ecc* arsenal and so it was highly likely that the *hor* mutant would prove less effective in causing soft rot when compared to the wild type strain. The profile of the infection for the *hor* mutant was not significantly different to that of the wild type *Ecc* strain in the first 50 hrs of infection. However after this time point there was a divergence in the two profiles and the *hor* mutant caused significantly less rot (approximately 50 % less compared to the wild type after 96 hrs) in the later stages of the experiment. This may be explained by the fact that only the Pel enzymes were greatly affected by the *hor* mutation, and so if there were any differences in the ability of the mutant to cause soft rot it would only become obvious later on in the infection. These results were consistent with previous reports (Murata *et al.*, 1991; Pirhonen *et al.*, 1991; Jones *et al.*, 1993) in that the co-ordinate down regulation of Pel, Cel and Prt had a deleterious effect on the ability of *Ecc* to cause soft rot. Caution must be taken when considering these results because variability of the data obtained from this type of crude *in planta*

assay has been reported previously (Jones, 1994). To improve these data, larger sample sizes would be needed.

The introduction of *hor* into the *Ecc* strains which had previously failed to express pigment produced mixed results. The *hor* gene was important for the expression of pigment in some strains of *Ecc*. However in the other strains the situation was more complex with other factors, still to be characterised, apparently required for the expression of prodigiosin in *Ecc*. This might have been anticipated because prodigiosin is not expressed in *E.coli* with *hor in trans* even with the addition of exogenous OHHL (data not shown). The failure to express pigment by some of the *Ecc* strains highlights the fact that there are significant regulatory differences even between highly related strains of the same species of bacteria.

The marker exchange mutant was dramatically reduced in its expression of pigment compared to the wild type *Ecc* strain, confirming *hor* to be involved with the expression of prodigiosin in *Ecc*. However, on closer observation there was a very pale pink colour to ATM101 colonies, perhaps suggesting that *hor* is still partially active in this strain, an idea mooted before as one of the possible reasons for the failure to complement the *hor* mutant *in trans* (section 7.3).

The data from the *lacZ* fusions to *carR* and *carI* indicated that the transcription of these genes was unaffected by the *hor* mutation. As previously reported (G. Bozgelmez and M. Rivet unpublished data) the expression of *carR* and *carI* were found to be constitutive in this strain of *Ecc*, which is different from *Photobacterium fischeri* where the homologue of *carI*, *luxI*, is induced between late log and early stationary phase. The results from *Ecc* strain GBH7 (*carA::lacZ*) were very different, with the maximal β -galactosidase activity of the *hor_{Ec}* mutant nearly 100 fold less than the *hor*⁺ strain GB7. This indicates that Hor must modulate the transcription of the carbapenem biosynthetic genes (*carA* at least). Addition of OHHL to the culture at varying times, resulted in a partial induction of the activity of the *carA::lacZ* fusion. Assuming that this would result in the production of carbapenem then this was consistent with the

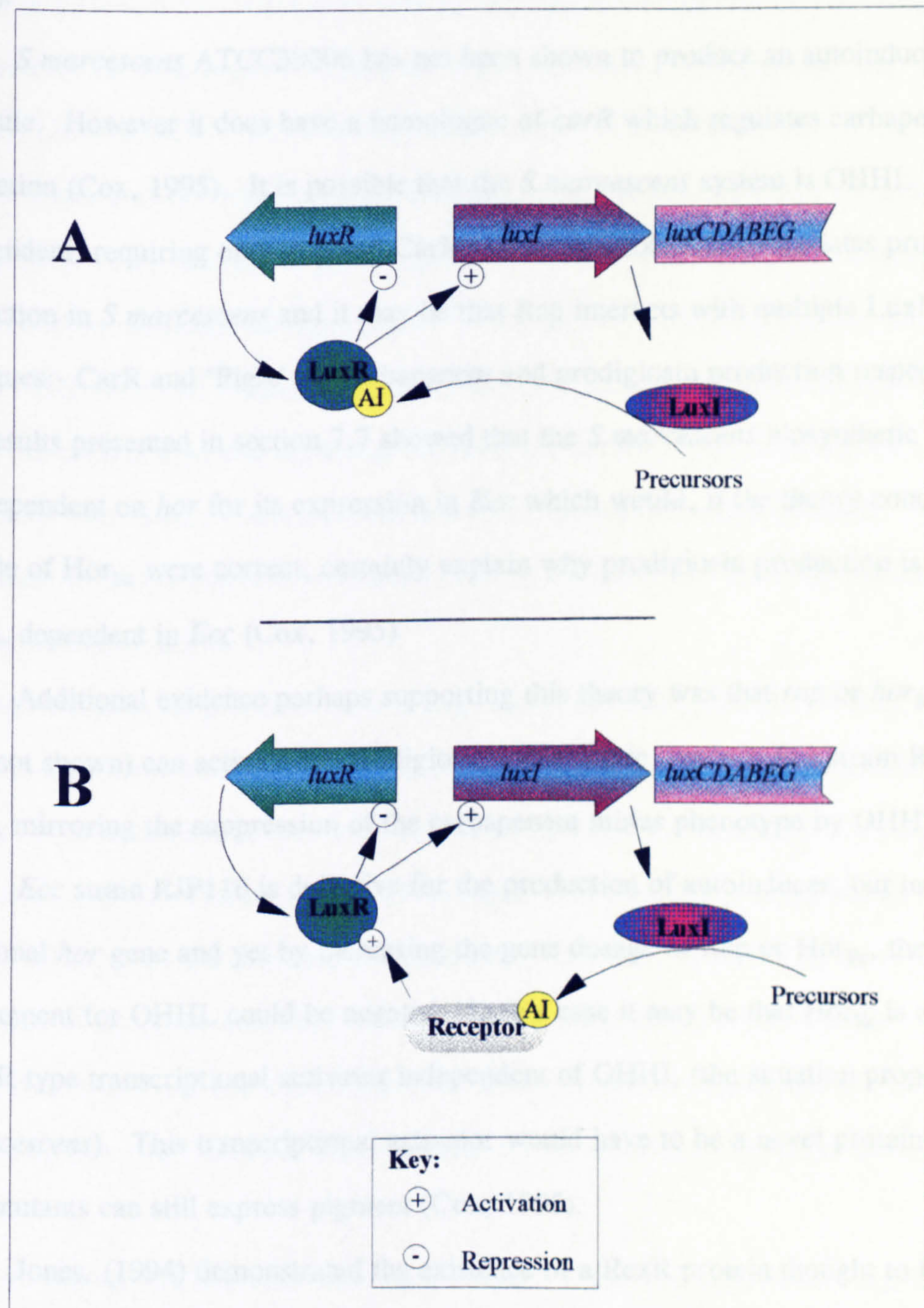
results from the bioassay in that suppression of the carbapenem null phenotype can be achieved by the exogenous addition of OHHL (section 7.2.2). The addition of OHHL resulted in an 8-10 fold increase in the β -galactosidase activity whether it was added at the start of the incubation or after 4 hrs, therefore it was likely that the OHHL was not limiting and the β -galactosidase activity recorded for these cultures represented the maximum Hor independent *carA* induction.

7.9 FINAL DISCUSSION

Before a possible role can be assigned to Hor it is important to consider what is known about this type of small molecule dependent regulatory system in other bacteria. Most of the research into the autoinducer paradigm has been focused on the *lux* system controlling bioluminescence in *Photobacterium fischeri*. This has been previously discussed in section 1.5.4 and so only the details pertinent to the possible role of Hor will be discussed here. There are three primary components for the regulation of bioluminescence in *P.fischeri*: *luxI*, *luxR* and the control region separating the two genes. LuxR is purported to be a transcriptional activator, the C-terminus of LuxR has a Helix-turn-Helix motif homologous to many DNA binding proteins. It is thought that LuxR binds the control region and activates the transcription of itself and the *lux* operon *luxICDABEG* (Choi and Greenberg, 1991) in an autoinducer dependent manner. *P.fischeri* produces three autoinducer molecules: N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), N-hexanoyl-L-homoserine lactone and N-octanoyl-L-homoserine lactone (see table 1.1). The synthesis of the first two of these molecules is directed by the product of the *luxI* gene. The bioluminescence is regulated by OHHL a molecule identical to the autoinducer found in *Ecc* which regulates carbapenem production. Much of the evidence so far points toward there being a direct interaction between OHHL and the N-terminal region of the LuxR protein (Hanzelka and Greenberg, 1995). The N-terminal domain of LuxR appears to block the DNA binding capacity of the C-terminal, it is thought that the autoinducer by binding to the N-terminal in some way relieves this

block on the C-terminal thereby allowing DNA binding and transcriptional activation. The precise mechanism of this activation is not known nor is its effect on the structure of the LuxR protein. Recent reports by Sitnikov *et al.*, (unpublished; cited in Sitnikov *et al.*, 1995) have put forward an alternative to the idea of a direct interaction between OHHL and LuxR. They report that N-decanoyl homoserine lactone an analogue of OHHL inhibits the *lux* system in *P.fischeri*, however this molecule will activate the *P.fischeri lux* system when it is cloned into *E.coli*. In the same way the *Vibrio harveyi* autoinducer will not activate the *lux* system in *P.fischeri* but will when it is cloned in *E.coli*. They put forward the idea that there is an additional protein(s) which is species specific in its autoinducer binding from *P.fischeri* and *V.harveyi*, but one that is more promiscuous in *E.coli* (depicted in figure 7.17); the precise mode of action of this protein is not known.

The findings of this study demonstrated that Hor was important for the expression of carbapenem and exoenzymes in *Ecc*. These systems are regulated by an similar system to *lux* (Jones *et al.*, 1993; McGowan *et al.*, 1995). It would appear that Hor does not affect the transcription of *carR* or *carI* but acts directly on *carA*, a biosynthetic gene. It was difficult to explain why in a strain which produces OHHL and had a functional *carR* gene, adding these elements in excess could suppress the Hor phenotype. The hypothesis put forward by Sitnikov *et al.*, (1995) may offer an explanation. The Hor protein may bring about or stabilise the proposed interaction between OHHL and *carR*, this in turn would allow the efficient binding of *carR* to DNA and or activate the transcription of the carbapenem operon. The observed suppression would then be explained by the following: at normal physiological levels of *carR* and OHHL, the Hor protein is a vital component for the activation of the *car* operon. OHHL and *carR* can interact, albeit inefficiently in the absence of Hor. However at physiological levels of these two elements this is insufficient to activate detectable levels of carbapenem production. As was observed, only when either element is in excess can significant Hor independent induction of carbapenem take place. If this hypothesis is

FIGURE 7.17Taken from Sitnikov *et al.*, (1995)**Figure 7.17 (A) A model for the cell-density-dependent regulation of luminous bacteria**

A model of the commonly accepted cell-density-dependent regulatory circuit of *Photobacterium fischeri* involving the direct interaction of autoinducer (AI) and LuxR. **(B)** An alternative model suggested by Sitnikov *et al.*, (1995), whereby the autoinducer is sensed by a receptor protein which in turn activates the LuxR protein. For simplicity regulation by CRP has been left off this diagram (see section 1.5.4).

correct then the SlyA related protein reported by Ludwig *et al.*, (1995) might function as the OHHL receptor protein in *E.coli* which was suggested to exist by Snitkov *et al.*, (1995).

S.marcescens ATCC39006 has not been shown to produce an autoinducer molecule. However it does have a homologue of *carR* which regulates carbapenem production (Cox, 1995). It is possible that the *S.marcescens* system is OHHL independent, requiring only Rap and CarR. The Rap protein also regulates prodigiosin production in *S.marcescens* and it may be that Rap interacts with multiple LuxR analogues:- CarR and 'PigR' for carbapenem and prodigiosin production respectively. The results presented in section 7.7 showed that the *S.marcescens* biosynthetic cosmid was dependent on *hor* for its expression in *Ecc* which would, if the theory concerning the role of Hor_{Ec} were correct, certainly explain why prodigiosin production is also OHHL dependent in *Ecc* (Cox, 1995).

Additional evidence perhaps supporting this theory was that *rap* or *hor_{Ec}* *in trans* (data not shown) can activate the prodigiosin biosynthetic genes in *Ecc* strain RJP116 (*carI*), mirroring the suppression of the carbapenem minus phenotype by OHHL and CarR. *Ecc* strain RJP116 is defective for the production of autoinducer, but has a functional *hor* gene and yet by increasing the gene dosage of Rap or Hor_{Ec}, the requirement for OHHL could be negated. In this case it may be that Hor_{Ec} is activating a LuxR type transcriptional activator independent of OHHL (the situation proposed for *S.marcescens*). This transcriptional activator would have to be a novel protein because *carR* mutants can still express pigment (Cox, 1995).

Jones, (1994) demonstrated the existence of a RexR protein thought to be responsible for the activation of exoenzymes (Regulation of Exoenzymes). This protein may interact with Hor in the same way to regulate exoenzyme production. The reason that there was not a complete cessation in exoenzyme production (as defined by indicator plate assays) maybe because only some of the isozymes require Hor for the activation of their expression. It should also be noted however that adding exogenous

OHL did not restore the wild type levels of enzyme production to the *hor* mutant (data not shown).

It is clear that the evidence to support the theory for this function of Hor_{Ec} and by implication the homologues of Rap, although interesting, is fragmented and essentially circumstantial. There are significant differences between the OHL regulated systems of *Ecc* and *P.fischeri* that have been characterised thus far and so this evidence does not by any means prove this notion for the function of the homologues of *rap*. In addition, although the homologues of Rap and SlyA define a distinct subgroup within a wider family of bacterial regulatory proteins, the theory presented here does not relate to the present evidence regarding the function of these other homologues.

Only with further research could less speculative conclusions be made. Possibilities for future research to address some of the questions raised by the results of this study are listed below.

- 1) It would be interesting to determine if Rap, Hor_{Ec} and Hor_{Ye} are able to bind DNA. Whilst this thesis was being written the *E.coli* SlyA homologue and the *Echr* PccS regulatory protein have been shown to be able to bind DNA (Oscarsson *et al.*, 1996; Praillet *et al.*, 1996). Praillet *et al.*, (1996) report that the DNA binding ability of PccS may be potentiated by the presence of unidentified factor(s). With this in mind, if the homologues of Hor_{Ec} are also able to bind DNA, and the theory relating to an interaction between Hor and CarR is correct, it would also be interesting to see if there was a synergistic effect between these proteins for the binding of DNA (or in fact any effect).
- 2) Because prodigiosin is produced in the transition phase and carbapenem is produced constitutively in *S.marcescens* it would be interesting to construct transcriptional fusions to *rap* to see when it is expressed. This would also serve as an assay with which to isolate possible regulatory elements higher up the regulatory cascade, i.e. those affecting the expression of the *rap* gene fusion.

- 3) In addition to the phenotypes described in this thesis there may be other global effects caused by the *rap/hor* mutations. By looking at the total protein profile of these mutants and comparing them with the wild type strain the true global nature of these regulators could be assessed.
- 4) It was unfortunate that there was insufficient time to investigate the function that the *hor* gene fulfils in *Yersinia enterocolitica*. *Yersinia* is known to make a pheromone which regulates both secondary metabolism and exoenzyme production and so future work would be directed at 'maker exchanging' the *Yersinia* homologue of *rap* as a useful comparison with *Ecc*. This would also be important if the *hor* genotype affected virulence of this important pathogen, for obvious reasons.

MAJOR FINDINGS

8.0 INTRODUCTION

At the inception of this study little was known about the production of carbapenem (Car) and prodigiosin (Pig) by *Serratia marcescens*. Prior to starting my PhD I had isolated various cosmids from a chromosomal library that were thought to contain; the carbapenem biosynthetic genes, the prodigiosin biosynthetic genes and a gene(s), denoted *rap* (regulation of antibiotic and pigment; carried on pNRT300), that could complement the pleiotropic antibiotic and pigment mutants back to Car⁺, Pig⁺.

The initial aim of this project was to develop a detailed genetic understanding of the regulation of antibiotic and pigment production in *S.marcescens* and to this end the major findings of this study are listed below:

8.1 MAJOR FINDINGS OF THIS STUDY

- i) The pigment encoded by cosmid pNRT104 was positively identified as prodigiosin. Of the bacteria tested only the erwinias were able to make prodigiosin when transformed with this cosmid. However, there were dramatic differences in the ability to express pigment between *Erwinia* strains and even between *Erwinia* cells within a bacterial colony (Chapter 3). This interesting observation raises the issue of control of differential gene expression within bacterial colonies, both in spatial and temporal terms. Unfortunately, there was simply not enough time to investigate these phenomena further. Nevertheless, I have provided the initial observation which could, and should, be the basis of a future research investigation.
- ii) The sequence of pNRT300 revealed four Orfs (two incomplete) and the gene products of two of these shared high levels of homology with SlyA, a virulence determinant from *Salmonella typhimurium*, and Pcp a lipoprotein found in *Yersinia enterocolitica* and *Haemophilus influenzae* (Chapter 4).

iii) Marker exchange analysis revealed that the *rap* gene was in fact the *slyA* homologue, casting serious doubts on the findings of Libby *et al.*, (1994) that SlyA was a haemolysin. The regulatory role has very recently been confirmed by Ludwig *et al.*, (1995) where SlyA was reclassified as a positive acting regulatory protein (at least in *Escherichia coli*). Unlike the two studies mentioned above this study also detected *rap* (and therefore *slyA*) homologues in various bacteria, and revealed virtual ubiquity in the erwinias (Chapter 5).

iv) The homologues of *rap* (*hor* genes), detected in *Erwinia carotovora* subspecies *carotovora* (*Ecc*) and *Yersinia enterocolitica*, were isolated and sequenced. This was partly facilitated by the conservation in the arrangement of genes bordering the *rap* and *hor* genes. The *rap* homologues isolated in this study define a subgroup of a wider family of newly discovered bacterial regulatory proteins, some of which have been shown to have some DNA binding abilities (Chapter 6).

v) The *Ecc hor* gene was shown to not only regulate carbapenem production but also exoenzyme synthesis, with the resultant diminution in virulence as demonstrated by reduced ability to cause soft rot. Some evidence was also provided which points towards an involvement of *hor* in the bacterial pheromone class of regulatory systems (Chapter 7).

The findings of this study have answered many of the initial questions relating to its aim. However it has also posed many more exciting questions which are now under investigation by others in this group.

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APPENDIX

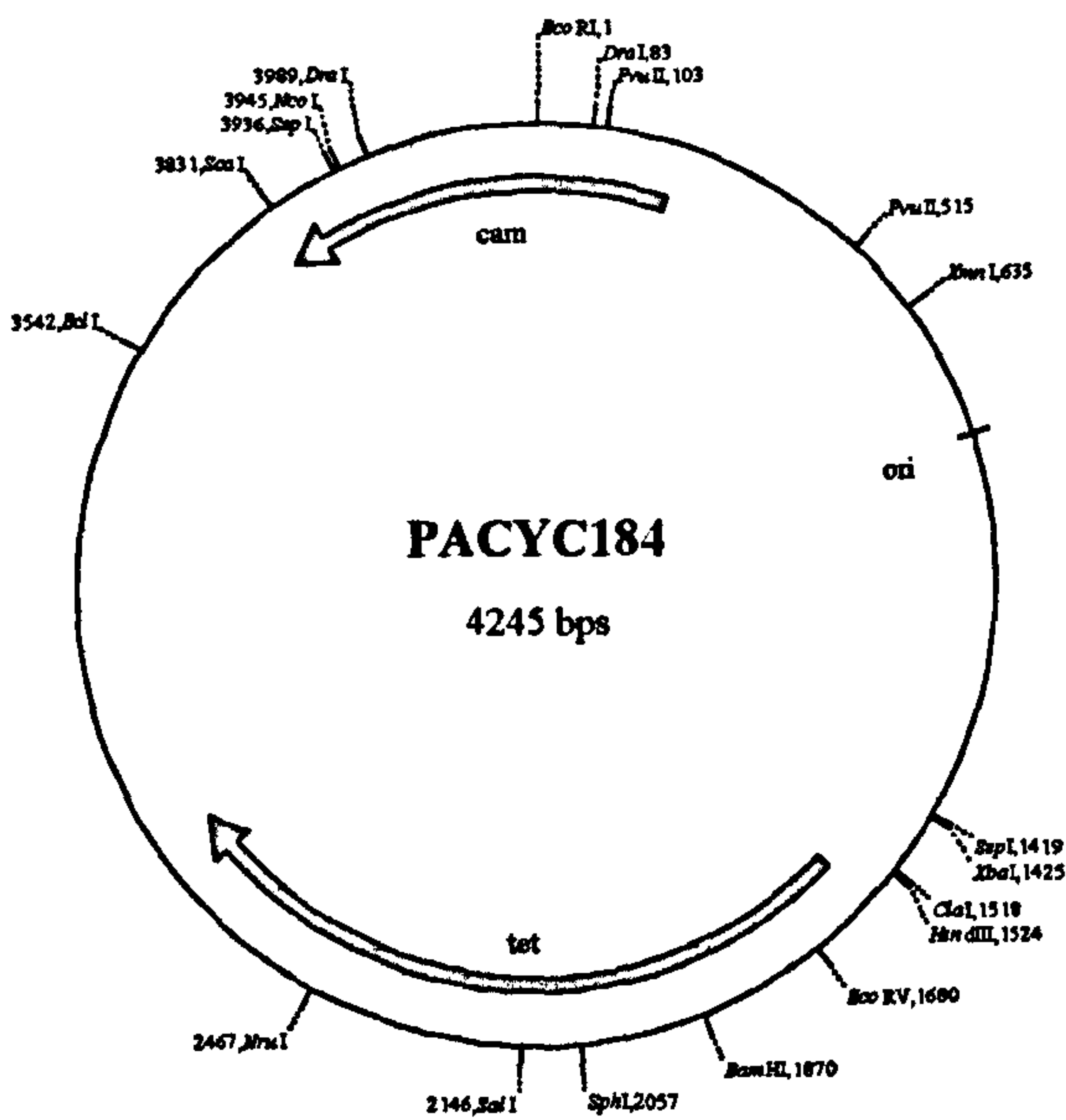
OLIGONUCLEOTIDE PRIMERS USED IN THE COURSE OF THIS STUDY

Name	Sequence (5'- 3')	Cycle Sequence	Restrictio Site	Optimum annealing temperature (°C) ^a
Cyto1	GCTAATTATAAGGAGTA	No	None	39
Cyto2	TTTCAAGTGCCATACAA	No	None	41
Cyclcyto1	<u>CCGAATTC</u> AGAGTTGATTGCAATCC	Yes	<i>Eco</i> RI	49
Cyclcyto2	<u>TAGGCCTC</u> GAATAATCAACTCCCG	Yes	<i>Stu</i> I	45
Cyclcyto3	<u>TAGGCCTA</u> ACACTCTGTTAATGGC	Yes	<i>Stu</i> I	43
PcpS1	<u>CCGAATTC</u> ATCGCCATTACAGGAG	No	<i>Eco</i> RI	43
PcpS2	<u>TAGGCCTC</u> GATTAATCACCCCTCG	No	<i>Stu</i> I	47
Erapfil	AGCTGTTGGGATGATGC	M13 ^b	None	-
CyclYen1	<u>CCATCGATT</u> CTGCTGTGAATTGAG	Yes	<i>Cla</i> I	43
CyclYen2	CGAATGATGGTTCGTTTCG	Yes	None	49
CyclYen4	CTCCAGTTGATCTACGGTTCT	Yes	None	55

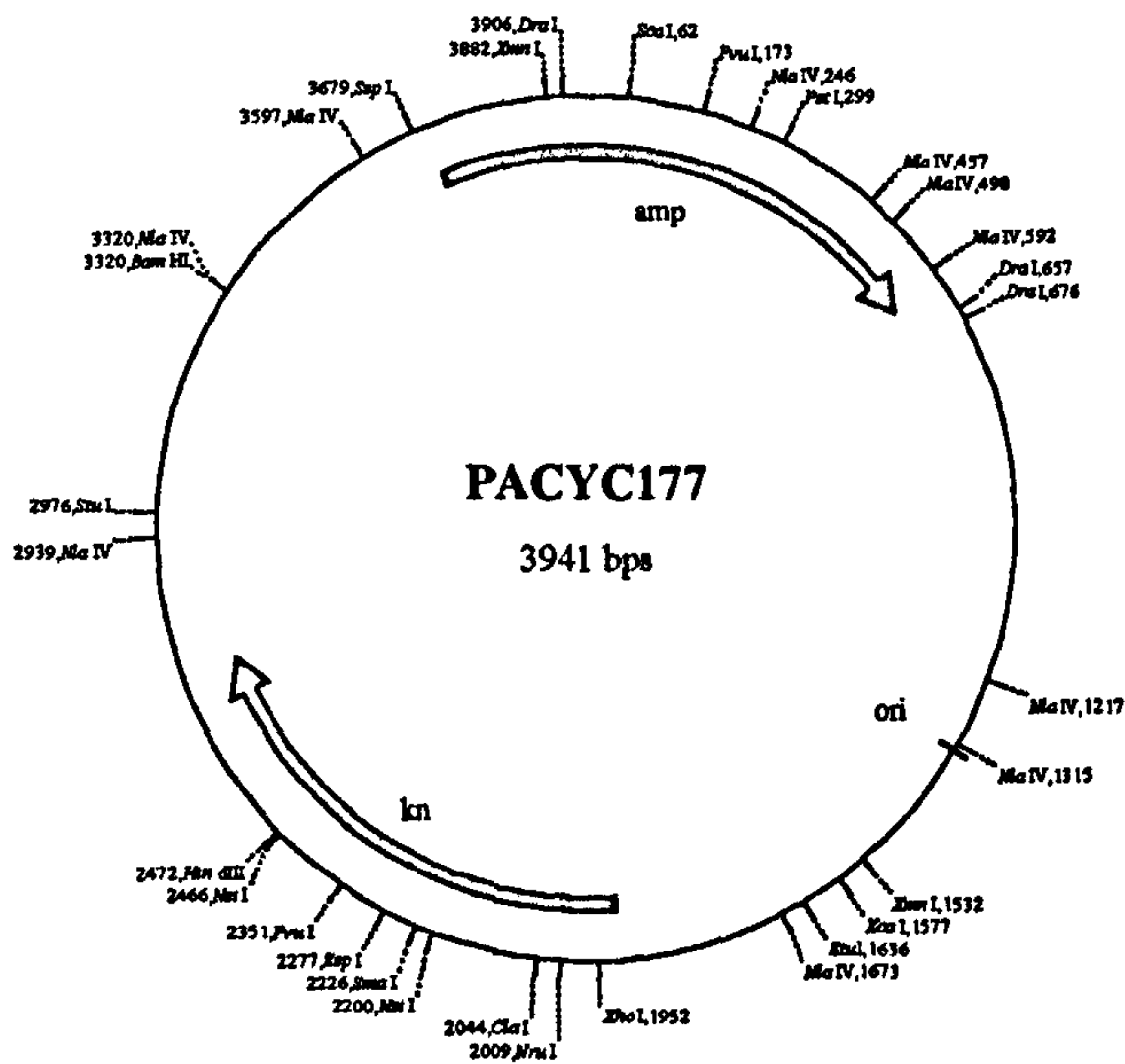
Key:

- a- calculated by the Wallace rule Ikatura *et al.*, (1984)
 - b- used for sequencing M13 template DNA.
- Restriction sites have been underlined.

A

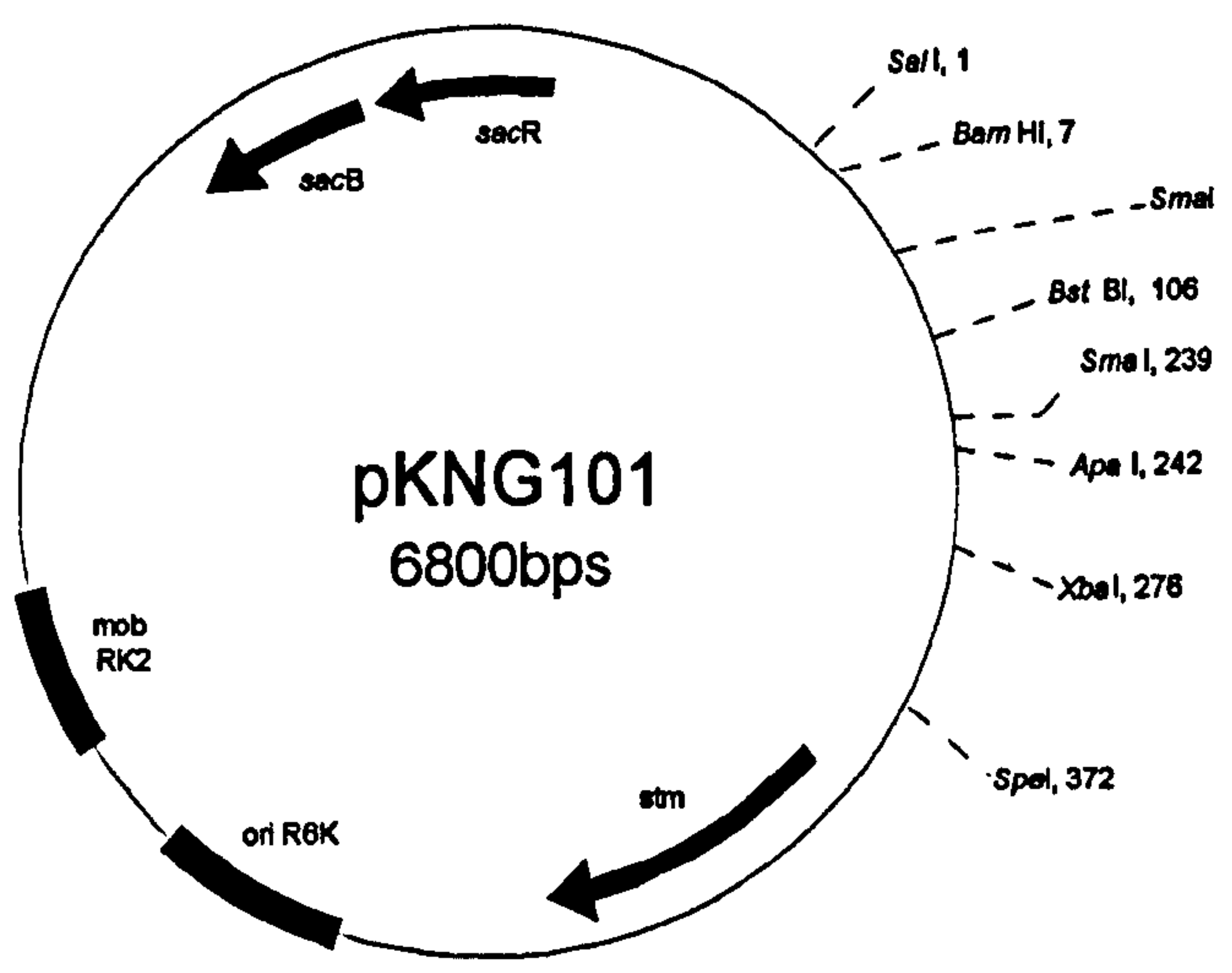


B

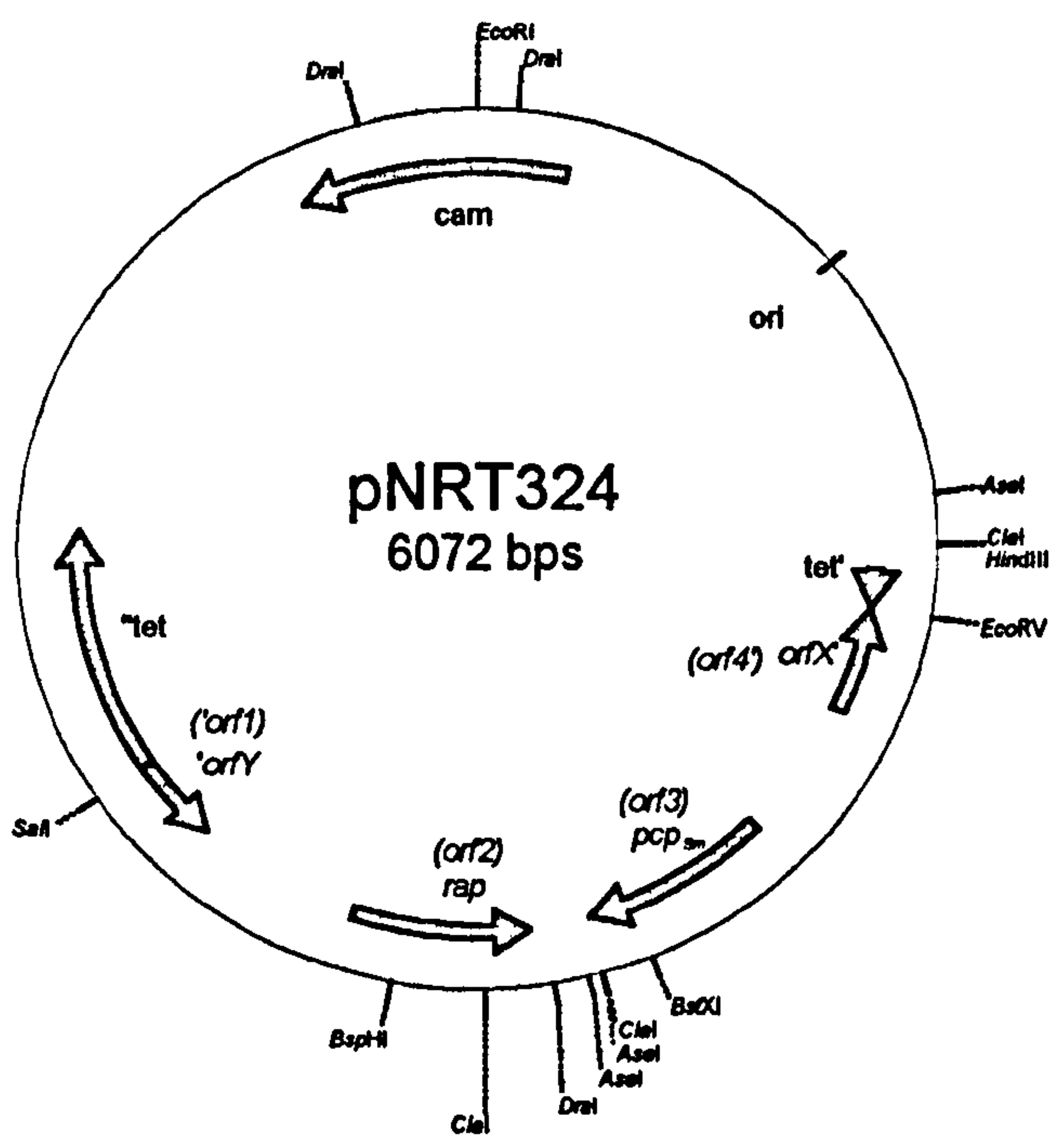


A + B The low copy cloning vectors pACYC184 (Cm^R , Tc^R) pACYC177 (Amp^R , Kan^R) 10/15 copies per chromosome (Chang and Cohen, 1978).

A

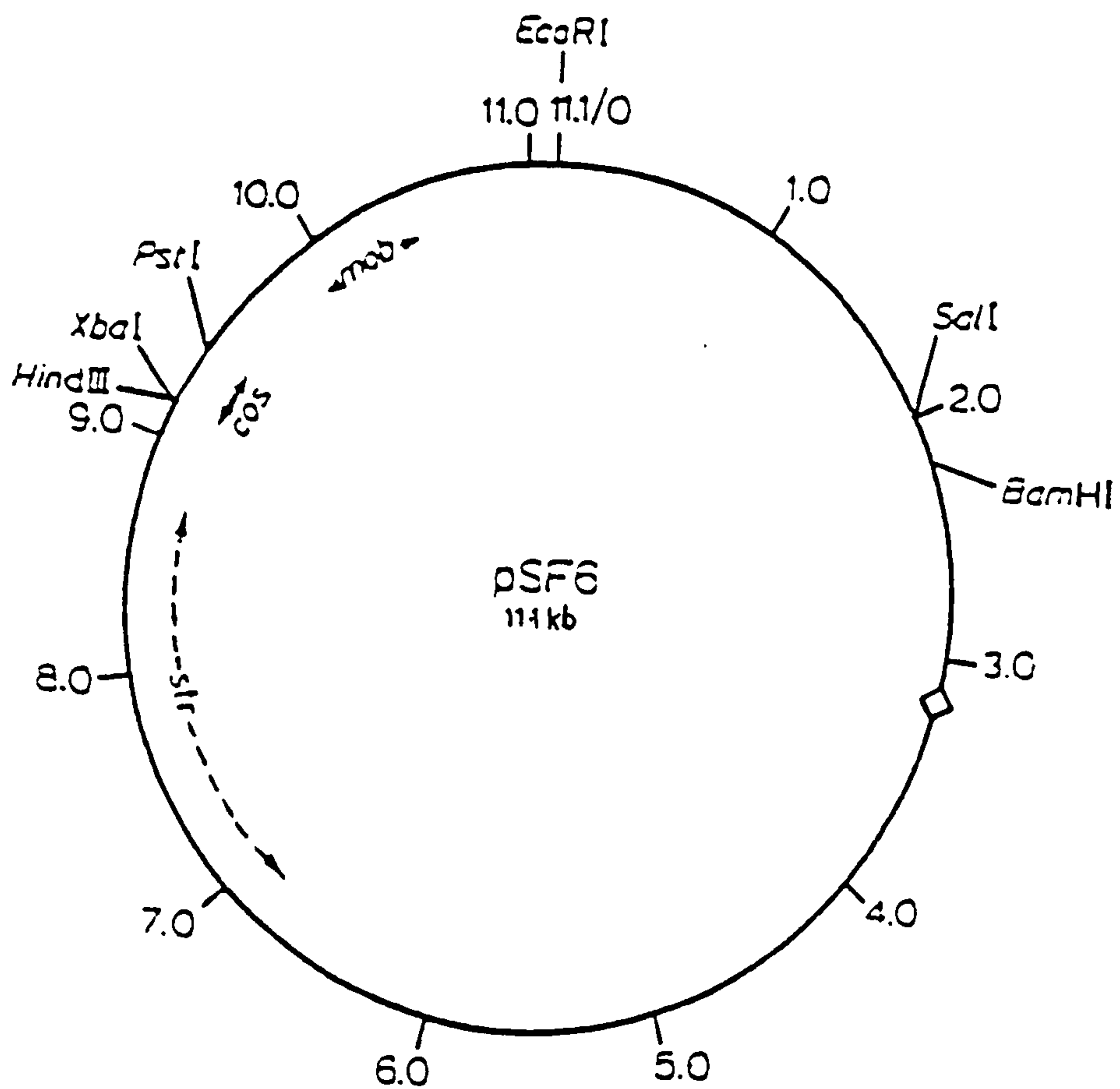


B



A - pKNG101 *sacB* marker exchange plasmid (Kaniga *et al.*, 1991).

B - *rap* gene containing subclone pNRT324 (this study), based on pACYC184. Gene names in brackets refer to temporary names given to the respective genes earlier in the study.



pSF6 - Low copy cloning vector carrying the cohesive site (*cos*) of bacteriophage λ and the *mob* (mobilisation) region of the broad host range plasmid RK2 (selvaraj *et al.*, 1984).